"SCIENTIFIC EVALUATION OF ANTIOXIDANT AND ANTI CANCER ACTIVITY OF KANCHANARA GUGGULU VATI BY *INVITRO* METHODS"

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

CHENNAI-600032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

Submitted by

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CERTIFICATE

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DETICATED

TO GOD &

MY FAMILY

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LIST OF ABBREVIATIONS

BAX	Bcl-2 associated X protein
BCI-2	B cell lymphoma 2
BRCA 1	Breast Cancer Susceptibility Protein
DCIS	Ductal Carcinoma In Situ
DMSO	Dimethyl Sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme- Linked Immunosorbent Assay
EDTA	Ethylene Diamine Tetra Acetate
FBS	Foetal Bovine Serum
FT-IR	Fourier- Transform Infrared Spectroscopy
GC-MS	Gas Chromatography- Mass Spectroscopy
IC50	Median Inhibition Concentration
IFFKG	Isolated Flavonoid Fraction of KancanaraGuggulu
IL	Interleukin
LCIS	Lobular Carcinoma In Situ
MCF-7	Michigan Cabcer Foundation-7

MTT	Dimethyl thiazolyldiphenyltetrazolium salt
MCEKG	Methanolic Crude Extract of kancanaraGuggulu
NCCS	National Center for Cell Science
P^{53}	Protein 53
PBS	Phosphate Buffer saline
RBC	Red Blood Cells
RPMI	Rose Well Park Memorial Institute Medium
TLC	Thin Layer Chromatography
TTE	Tris-Taurine-EDTA buffer
TPVG	Trypsin, PBS, Versene/EDTA, Glucose
WBC	White Blood Cells
WHO	World Health Organisation

1. INTRODUCTION

Cancer is one of the dreadful diseases of 20th century and moving vastly towards 21st century. According to the studies, worldwide about 6 million new incidences are reported every year. It is the second major cause of death after cardiovascular diseases.[1]

'Neoplasm' or tumor is a mass of tissue formed as a result of abnormal, excessive, uncoordinated, autonomous and purposeless proliferation of cells even after cessation of stimulus for growth which caused it.

The branch of science dealing with the study of neoplasms or tumours are called oncology *(oncos*= tumour, *logos*= study). Neoplasms may be a **'benign'** when they are slow-growing and localised without causing much difficulty to the host, or **'malignant'** when they proliferate rapidly, spread throughout the body and may eventually cause death of the host. The common term used for all malignant tumours is **cancer.** [2]

1.1 HISTORY

The cancer is existing from all of our human history; the earliest written record of cancer is from 3000 BC by the Egyptian Edwin Smith papyrus who described about breast cancer.

The several types of cancer were described with Greek word 'carcinos' (crab or cray fish) by Hippocrates between 460 BC and 370 BC. The Greek Celsus between 25 BC and 50 AD translated the term 'carcinos' in to 'cancer' (crab) in Latin term and recommended surgery as treatment.[3]

In the 15th to 17th centuries doctors discovered the cause of the death by dissecting the bodies.[4] With the help of microscope 'cancer spread', metastasis was discovered by English surgeon Campbell De morgan between 1871 and 74. In the 19th centuries, Marie

curie and Pierre curie discover radiation, was used for effective non- surgical treatment for cancer.

The first use of drugs to treat cancer in the early 20th century was "Mustard gas" but its side effects, decreasing WBC count, was unacceptable. So, researchers looked for other substances and discovered 'Mustine'. Since then several drugs have been developed to treat cancer.[5]

1.2 CURRENT STATISTICS OF CANCER WORLDWIDE

Worldwide cancer incidence and mortality statistics are taken from the International Agency on Cancer GLOBAGAN database and the World Health Organisation, Global Health Observatory and the United Nations World Population Prospects report.[6]

- In 2008 approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non- invasive cancers). In 2012, an estimated 14.1 million new cases of cancer occurred worldwide. More than half of cancers occurring are in less developed regions.
- The four most common cancers occurring worldwide are prostate, bowel, female breast and lung cancer. These four cancers account for around 4 in 10 of all cancers diagnosed worldwide.
- In 2010 nearly 7.98 million people died with cancer. In 2012, estimated 8.1 million people died from cancer worldwide. More than 6 in 10 cancer deaths worldwide occur in less developed regions of the world.[7]

1.3 CANCER IN INDIA

Cancer rates in India are considerably lower than those in developed countries such as United States data from population based cancer registries in India show that the most

frequently reported cancer sites in males are lung, oesophagus, stomach, and larynx. In females, cancers of the cervix, breast, ovary and oesophagus are the most commonly encountered.[8]

- India officially recorded over half a million deaths due to cancer in 2011-5.3 lakhs as against 5.24 lakhs in 2010 and 5.14 lakhs in 2009.
- The estimated number of new cancers in India per year is about 7 lakhs and over 3.5 lakhs people die of cancer each year. Cancer is the leading cause of death worldwide, accounting for 7.6 million deaths in 2008.
- Breast, lung, liver, stomach, colon cancer cause the most cancer deaths each year.
- Death from cancer worldwide are projected to continue rising with an estimated 13.1 million deaths in 2030.
- The risk of developing cancer generally increases with age and mass lifestyle changes occur in the developing world.[5]

1.4 BREAST CANCER

Breast cancer is a malignant neoplasm of breast tissue, either in cells that line the ducts that carry milk to the nipples (ducts cancer) and/or in cells that line the lobules that involved in milk production (lobular cancer). Tumors of the female breast are common and clinically significant but rare in men.

Among the important benign tumors are fibroadenoma, phyllodes tumor (cystosarcomaphyllodes) and intraductal papilloma.

Carcinoma of the breast is an important malignant tumor which occurs as non – invasive (carcinoma in-situ) and invasive cancer with its various morphological varieties.[9]

1.5 INCIDENCE OF BREAST CANCER

As per GLUBAGON data, the breast cancer in women in the US are most common, about 1 in 8 have chance of developing breast cancer in their lifetime, but in India the overall incidence is less than US, but it's not far behind, because in 2008 the incidence rate was 182460 in US whereas 115251 new cases were diagnosed in India, it shows the cancer burden in India is almost reached about 2/3 of that of the US and is raising steadily.

Since cancer deaths is projected to continue rising with an estimated 13.1 million deaths in 2030, particularly breast cancer accounting for 23% incidence and 13.7% deaths worldwide and mortality rate is expected to increase in India as per GLOBAGAN 2008 data.[10]

1.6 PREVENTION OF CANCER

In recent years, certain precautionary measures are advocated to prevent or reduce the occurrence of cancer. The most important among them from the biochemical perspective, are **Antioxidants** namely vitamin E, ß-carotene, vitamin C and selenium.

The anti-oxidants prevent the formation or detoxify the existing free radicals (free radicals are known to promote carcinogenesis). In addition antioxidants are stimulate immune system, and promote detoxification of various carcinogens.

In general, most of the vegetables and fruits are rich in antioxidants. Their increased consumption is advocated to prevent cancer.[11]

1.7 ROLE OF FREE RADICALS IN CANCER

A **free radical** is defined as a molecule or a molecular species that contains one or more unpaired electrons, and is capable of independent existence (E. g) O_2^-, OH^-, COO^-

Reactive oxygen species are a type of unstable non-radical derivatives of O_2 which do not contain unpaired electron and that easily react with other molecules in a cell (e. g) H_2O_2, $^1O_2$. The term reactive oxygen species are used in broad sense to collectively represent free radicals and (non-free radicals which are extremely reactive) of biological system.

1.7.1 SOURCES AND GENERATION OF FREE RADICALS

The major sources responsible for the generation of free radicals may be considered under two categories

1. Due to normal biological processes (or cellular metabolism).
2. Due to environmental effects.

Cellular metabolism

- Leakage of electrons from the respiratory chain (ETC)
- Production of H_2O_2 or O_2^- by oxidase enzymes (e .g) Xanthine oxidase, NADPH oxidase).
- Due to chain reactions of membrane lipid peroxidation.
- During the synthesis of prostaglandins.
- Production of nitric oxide from arginine.
- During the course of phagocytosis (as a part of bacterial action).
- In the oxidation of heme to bile pigments.
- As a result of auto-oxidation e.g. metal ions [$Fe^{2+,}$ Cu^{2+}]; ascorbic acid, glutathione, flavin coenzymes.

Environmental effects[12]

- As a result of drug metabolism e.g. paracetamol, halotahane, cytochrome P_{450} related reactions.
- Due to damages caused by ionizing radiations (e. g. X-rays) on tissues.
- Photolysis of O_2 by light.
- Photoextitation of organic molecules.
- Cigarette smoke contains free radicals, and trace metals that generate $OH^{\cdot}$.
- Alcohol promote lipid peroxidation.

1.7.2 FREE RADICALS MECHANISM INVOLVED IN CANCER PATHOGENESIS

Cancer development can be described by three stages:

- **Initiation**
- **Promotion**
- **Progression**

ROS can act in all these stages of carcinogenesis which ultimately leads to cancer by following mechanisms. [13]

a) Free radicals can damage DNA, and cause mutagenicity and cytotoxicity, and thus play a major role in carcinogenesis.
b) It is believed that ROS can induce mutations, and inhibit DNA repair process, that results in inactivation of certain tumor suppressor genes leading to cancer.
c) Further, free radicals promote biochemical and molecular changes for rapid growth of tumor cells.[14]

1.8 ANTIOXIDANTS

The term “antioxidants” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells.[13]A biological antioxidants may be defined as a **substance** (present in low concentration compared to an oxidizable substrate) that **significantly delays** or **inhibits oxidation** of a substrate.[14]

Humans have evolved highly complex antioxidant systems (enzymatic and non-enzymatic), which work synergistically, and in combination with each other to protect the cell and Organ systems of the body against free radical damage. The antioxidants can be either endogenous or exogenous in nature. They can be classified into three main categories:

1) The first line defense antioxidants which include superoxide dismutase (SOD), catalase, glutathione reductase (GR), minerals like Se, Cu, Zn etc.
2) The second line defense antioxidants which include glutathione (GSH), vitamin C, albumin, vitamin E, carotenoids, flavonoids etc.
3) The third line defense antioxidants which include a complex group of enzymes such as Lipase, Protease, DNA repair enzymes etc.

Antioxidants stabilize polyunsaturated fatty acids in foods by reacting with free radicals, chelating metal ions and interrupting the propagation phase of lipid peroxidation. Some antioxidants can interact with other antioxidants regenerating their original properties; this mechanism is often referred to as the “antioxidants network”.

There is growing evidence to support a link between increased levels of ROS and disturbed activities of enzymatic and non-enzymatic antioxidants in diseases associated with aging.

1.9 FLAVONOIDS

These are a broad class of low molecular ubiquitous groups of plant metabolite and are an integral part of the human diet. **Flavonoids** are **benzo-gamma-pyrone derivatives** consisting of phenolic and pyrane rings and during metabolism hydroxyl groups are added, methylated, sulfated or glucorinated.

There is intense interest in flavonoids due to their antioxidant and chelating properties and their possible role in the prevention of chronic and age related diseases.

Flavonoids are present in food mainly as glycosides and polymers and these comprise a substantial fraction of dietary flavonoids. The biological properties of flavonoids are determined by the extent, nature, position of the substituents and the number of hydroxyl groups.

These factors also determined whether a flavonoid will act as an antioxidant or as a modulator of enzyme activity, or whether it possesses anti-mutagenic or cytotoxic properties. The most reported activity of flavonoids is their **protection against oxidative stress.**

Thus flavonoids can scavenge peroxyl radicals and are effective inhibitors of lipid peroxidation, and can chelate redox-active metals, thus prevent catalytic breakdown of hydrogen peroxide (Fenton chemistry).[13]

1.9.1BREAST CANCER AND FLAVONOIDS – A ROLE IN PREVENTION

Endogenous estrogens, such as 17ß-estrodiol (E2), are implicated in the development of breast cancer. The putative mechanisms by which estrogens exert the carcinogenic effects have been recognized to involve the redox cycling of estrogen metabolites and subsequent estrogen-DNA adduct formation as well as the estrogen receptor-dependent pathway of

estrogen-induced cell growth. The former mechanism is regulated by phase I enzymes, mainly cytochrome P450 (CYP) 1A1, 1A2, and 1B1.

Epidemiological studies suggest an inverse association between a higher intake of flavonoids and breast cancer risk. Flavonoids, which are widely distributed in plant kingdom, have been recently reported as candidate compound that can exert chemopreventive effects in estrogen-dependent or independent breast cancer. [15]

Some flavonoids have been reported as potent aromotase inhibitors. Substantial evidence supports the concept that estrogens be involved in mammary carcinomas. Estradiol, the most potent endogenous estrogen, is bio synthesized from androgens by the cytochrome P450 enzyme complex called aromatase. Inhibition of aromatase is an important approach for reducing growth stimulatory effects of estrogens in hormone – dependent breast cancer. Therefore, flavonoids could be considered potential agents against breast cancer through the inhibition of aromatase activity. [16]

1.10 SIDE EFFECTS OF CONVENTIONAL CHEMOTHERAPHY [17]

Majority of the cytotoxic drugs have more profound effect on rapidly multiplying cells, because the most important target of action are the nucleic acids and their precursors and acid synthesis occurs during division, it causes following side effects.

- Bone marrow depression
- Lymphocytopenia
- Oral cavity infection
- Diarrhoea
- Alopecia
- Carcinogenicity
- Teratogenicity

- Hyperuricemia
- Oligozoospermia
- Impotence in males
- Immune depression

1.11 ROLE OF MEDICINAL PLANTS

In both developed and developing countries, breast cancer is the most frequent cancer and most frequent cause of deaths. The report, by National Confidential Enquiry into Patient Outcome and Death (NCEPOD) have shown that more than 1 in 4 patients died from the side effect rather than from cancer and patients suffering from treatment related toxicity despite receiving other treatment to reduce chemotherapy side effects. An another study discovered that breast cancer patients receiving chemotherapy, some parts of their brain, responsible for learning and memory was affected when compared to untreated patients.

In search of new agents to treat cancer with fewer or less side effects a number of medicinal plant has been evaluated, because in pharmacology several active ingredients are found from medicinal plants. Some of the successful and potential lead molecules isolated from medicinal plants are vincristine, vinblastine, taxol, campothecin and podophyllotoxin.

Since the medicinal plants have phytoconstituents, which protects the plants from oxidative damages and may have same role in humans. They have wide range of action such as antitumor, antiviral, antibacterial, antimutagenic etc. they may act in different stages of the development of malignant tumor by protecting the DNA from oxidative damages.

They inactivate carcinogen by inhibiting the expression of mutagenic genes, they also inactivate the enzymes charged with activating procarcinogens and activate the systems responsible for the detoxification of xenobiotics. It can also inhibit anti-apoptotic gene as well as activate apoptotic gene. [18]

1.12 KANCHANARA GUGGULU VATI

Kanchanar guggulu vati is also known as kancanara guggul is a polyherbal ayurvedic formulation is beneficial in hypothyroidism, PCOS, lipoma, weight loss, tumour, cancer, cysts,goiter, wounds, fistula, boils and skin disorders.[19]

It is claimed to have anti-oxidant, anti-cancer activity since these activities have not been scientifically proved, I have undertaken this study to evaluate the anti-oxidant and anti-cancer effect of Kanchanara guggulu vati (polyherbal formulation) by *In-vitro* methods.

2. AIM AND OBJECTIVES OF THE STUDY

- Preliminary phytochemical analysis of the polyherbal formulation “Kanchanara guggulu vati”.
- To evaluate the antioxidant activity (DPPH method) of polyherbal formulation “Kanchanara guggulu vati”.
- To evaluate the IC50 concentration of methanolic crude extract of “Kanchanara guggulu vati” by *in- vitro* cytotoxicity method (MTT assay).
- To isolate the flavonoid fraction from polyherbal formulation “Kanchanara guggulu vati” by column chromatography.
- Characterization of isolated flavonoid fraction by FT-IR and GC-MS.
- To evaluate the IC50 concentration of isolated flavonoid fraction of Kanchanara guggulu vati by *in- vitro* cytotoxicity method (MTT assay).
- To determine the DNA fragmentation activity of isolated flavonoid fraction from “Kanchanara guggulu vati”.

3. REVIEW OF LITERATURE

3.1 BREAST CANCER

Breast cancer is a malignant neoplasm of breast tissue, either in cells that line the ducts that carry milk to the nipples (ducts cancer) and/or in cells that line the lobules that involved in milk production (lobular cancer). Breast cancer may be benign or malignant. Benign tumor is not life threatening and non-invasive but, malignant tumor is life threatening and invasive.[9]

3.2 GENERAL FEATURES AND CLASSIFICATION

Carcinoma of the breast arises from the ductal epithelium in 90% cases while the remaining 10% originate from the lobular epithelium, breast cancer occurs more often in left breast than the right. Presents different types of carcinoma of the breast as proposed in the WHO classification with some modification. The important morphological forms are as follows.

A. NON-INVASIVE (IN SITU) CARCINOMA

1. Intra ductal carcinoma
2. Lobular carcinoma (in-situ)

B. INVASIVE CARCINOMA

1. Infiltrating (invasive) duct carcinoma-NOS (not otherwise specified)
2. Infiltrating lobular carcinoma
3. Medullary carcinoma
4. Colloidal (mucinous) carcinoma
5. Papillary carcinoma
6. Tubular carcinoma
7. Adenoid cystic
8. Secretary (juvenile) carcinoma

9. Inflammatory carcinoma
10. Carcinoma with metaplasia

C. **PAGET'S DISEASE OF THE NIPPLE**

3.2.1 NON-INVASIVE (IN SITU) CARCINOMA

In general, two types of non-invasive or in-situ carcinoma they are intra ductal carcinoma and lobular carcinoma (in-situ), are characterized histologically by presence of tumor cells within the ducts or lobules respectively without evidence of invasion.

3.2.2 INVASIVE CARCINOMA

Infiltrating duct carcinoma- NOS (not otherwise specified) is the classic breast cancer and is the most common histological pattern accounting for 70% cases of breast cancer. In fact, this is the pattern of cancer for which the terms 'cancer' and 'carcinoma' were first coined by Hippocrates. Clinically, majority of infiltrating duct carcinomas have a hard consistency due to dense collagenous stroma (scirrhous carcinoma). Retraction of the nipple and attachment of the tumor to underlying chest wall may be present.

3.2.3 PAGET'S DISEASE OF THE NIPPLE

Paget's disease ofthe nipple is an eczematoid of the nipple, often associated with an invasive or non-invasive ductal carcinoma of the underlying breast. Morphological features are the skin of the nipple and areola is crushed, fissured and ulcerated with oozing of serosanguineous fluid from the erosions. Histologically, the skin lesion is characterized by the presence of Paget's cells singly or in small clusters in the epidermis.

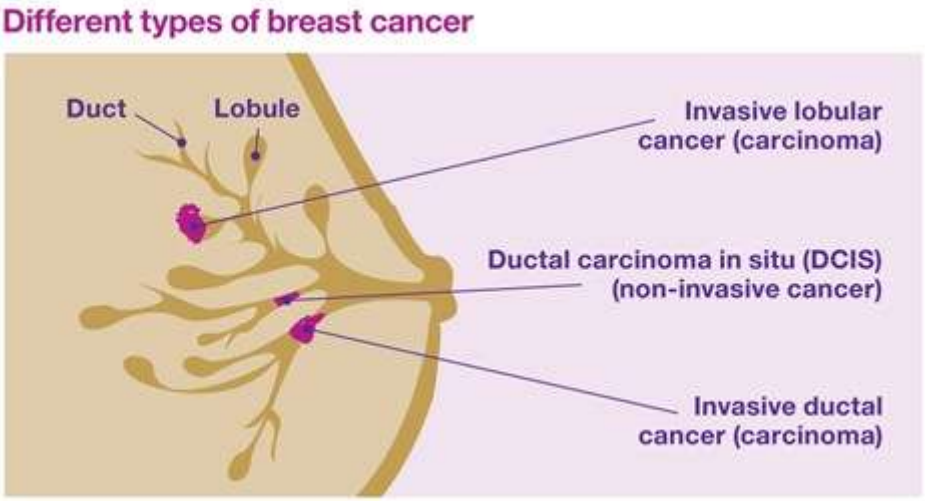


Figure: 1 Types of breast cancer

3.3 ETIOLOGY

Age: These incidence rises throughout a women's lifetime, peaking at the age of 75-80 years and then declining slightly thereafter. Breast cancer is very rare in before the age of 25.

Personal history: Women with atypical hyperplasia, lobular carcinoma in situ or ductal carcinoma in situ are more likely to develop invasive breast cancer.

First-degree relatives with breast cancer: A family history of breast cancer in the mother, father, sister or daughter increases the risk of breast cancer and the risk is even stronger if the family member was diagnosed before the age of 50 years old and/or the with pre-menopausal breast cancer.

BRCA1/BRCA2: Having mutations in BRCA1, a gene on chromosome 17 that controls cell growth or BRCA2, a gene on chromosome 13 that suppresses cell growth, are associated with a 40-80% increased risk of breast cancer.

Menstrual history: ages at menarche and menopause: Women who have an early age at menarche (<12 years) have a 30% increased risk of breast cancer while those who have a late age at menopause (>60 years) will have a 20-50% increased risk of disease.

Breast density on mammogram: Women with higher breast density have a higher risk of breast cancer.

Atypical Hyperplasia: A history of prior breast biopsies, especially if revealing atypical hyperplasia, increases the risk of invasive carcinoma. There is a smaller associated with proliferative breast changes without atypical hyperplasia.

Age at first live birth child: Women who experience a first a first full term pregnancy at the ages younger than 20 years have half of the re age of risk of nulliparous women or women over the age of 35 at their first birth. These associations are consistently observed for hormone receptor-positive breast cancer.

Race/ Ethnicity: Non-Hispanic white women have the highest rates of breast cancer. However, women of African or non-Hispanic ancestry present at a more advanced stage and have an increased mortality rate. Social factors such as decreased access to health care and lower use of mammography may well contribute to these disparities, but biological differences also play an important role.

Estrogen exposure: Women who have taken menopausal hormone therapy (estrogen + progestin for at least 5 years) have a 20% greater risk of developing breast cancer.

Radiation Exposure: Radiation to the chest, whether due to cancer therapy, atomic bomb exposure, or nuclear accidents, results in a higher rate of breast cancer. The risk is greatest with exposure at young ages and with high radiation doses.

Exercise: There is a probable small protective effect for women who are physically active.

Breastfeeding: The longer women breastfeed, the greater the reduction in risk. Lactation suppresses ovulation and may trigger terminal differentiation of luminal cells. The lower incidence of breast cancer in developing countries largely can be explained by the more frequent and longer nursing of infants.

3.4 SYMPTOMS OF BREAST CANCER [20]

- A lump or area of thickened tissue in either breast
- A change in the size or shape of the breast
- Discharge from the either of the nipple (which may be streaked with blood)
- A lump or swelling in either of the armpits
- Dimpling on the skin of the breast
- Pain in either of the breast or armpits not related to period
- Pus formation and foul odour
- Unusual loss of weight

3.5 PATHOGENESIS

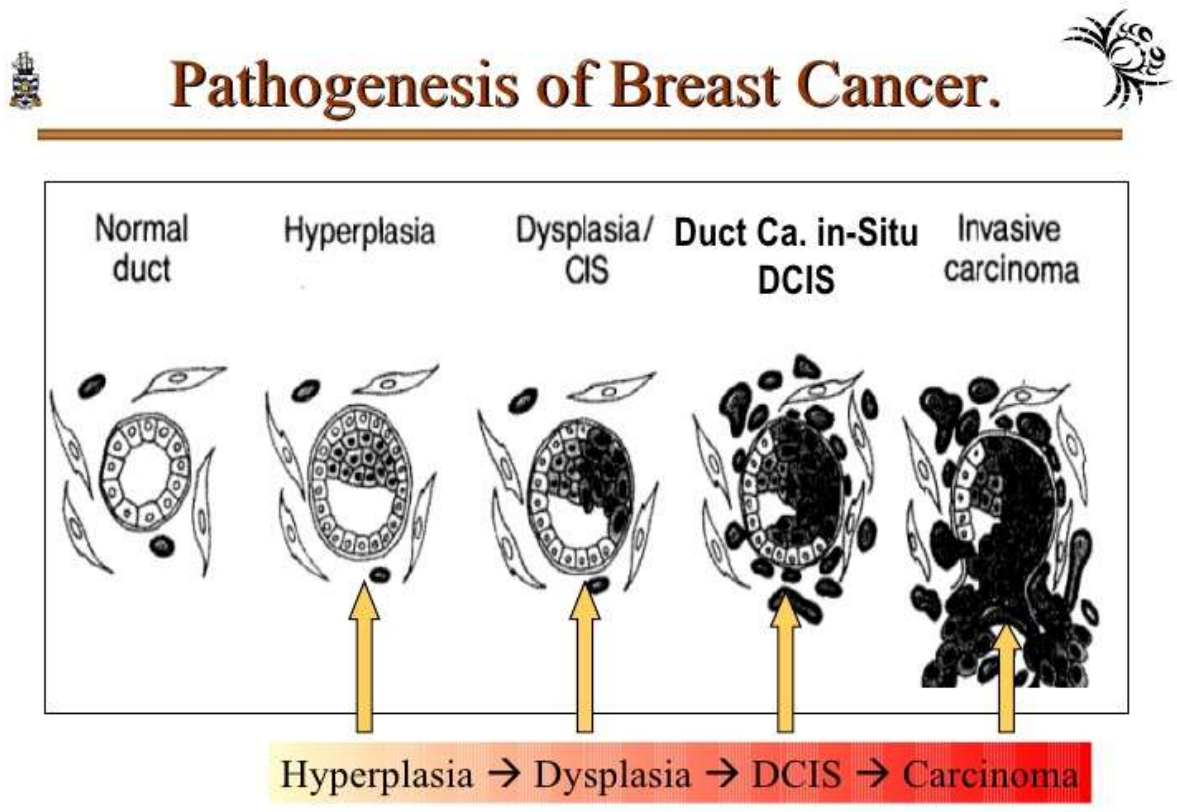


Figure: 2 Pathogenesis of breast cancer

The cells of breast tissue undergo abnormal proliferation and differentiation is due to some factors like, predisposing factors (gender, age and genetics), precipitating factors (diet, oral contraceptives and immune suppression) and etiology (unknown). It is lead to the following,[21]

- Genetic mutation of cellular DNA e.g.p53 (apoptotic protein), BRCA1& BRCA2 (tumor suppressor protein), which leads to activation of oncogenes. Inactivation of tumor suppressor gene, which leads to proliferation and differentiation of cancer cells in the milk duct and growth of malignant tumor

3.5.1 Growth of malignant tumor

- Activate immunologic mechanism which releases B-lymphocytes and T-lymphocytes, natural killer cells leads to cancer cell death.
- Release of growth factor which induce angiogenesis, supports tumor growth
- Cancer cells destroy normal RBCs, decrease HB, and nail beds become pale.

3.5.2 Rapid growth of malignant tumor

- Change in size of breast.
- Rapid multiplication of cancer cells, so the metabolism in the cancer cells will increase.
- The normal cells will be shortage of nutrients leads to unusual loss of weight and fatigue.
- Obstruct the milk duct cause rupture of it, so releases of inflammatory mediators such as bradykinin, prostaglandins cause transient vasoconstriction.
- Compression of blood and lymphatic vessels leads to decease blood supply to breast tissue cause ischemia followed by necrosis.
- Blood and lymph stasis increases the vascular permeability after vasodilatation; plasma fluid leak in to the tissues causes swelling.

- Swelling of breast tissue will compress the nerve endings produce pain and accumulate the fluid cells, cellular debris, live and dead lymphocytes will form PUS followed by tissue decay and foul odour will produce.
- Cancer cells begin to spread locally via lymphatic vessels and form tumor in axillary lymph nodes and supra clavicular lymph nodes which are palpable, immovable, hard and painless nodes.

3.5.3 Mutation of BRCA gene

Those individuals inherit defects in the DNA and gene or mutation of genes like BRCA1, BRCA2 are having more chances to develop breast cancer.

- **BRCA1,** it is a tumor suppressor gene, which produces a protein, called breast cancer type 1 susceptibility protein. The protein encoded by the BRCA1 gene combines with other tumor suppressors, DNA damage sensors, and signal transducer to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). It is found in the cells of breast and other tissue, where it helps repair of DNA double strand break, or induce Apoptosis.
- **BRCA2** is a tumor suppressor protein encoded by the BRCA2 gene. It is involved in the repair of chromosomal damage with an important role in the error- free repair of DNA double strand breaks.

3.5.4 Suppression of apoptotic signals

P53 is a protein with molecular weight of 53 KDa. This protein is encoded by TP53 gene and having tumor suppressing function.[22 ,23, 24] P53 can activate the repair protein to repair the damaged DNA, if fail to repair the damaged DNA, P53 can also arrest the cell cycle by holding at G1/S phase. It is also plays a major role in apoptosis, genomic instability, and inhibition of angiogenesis. In cancer, mutation occur at DNA Binding Domain (DBD) so

the ability of this protein to bind to its target DNA sequences is inhibited, and thus prevents transcriptional activation of these genes.

TNFα the primary role of TNF is in the regulation of immune cells. TNF was thought to be produced primarily by macrophages, but it is also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and neurons. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, sepsis (through IL1 & IL6 production), cachexia, inflammationand to inhibit tumorigenesisand viral replication. Dysregulation of TNF production is associated at variety of human diseases including Alzhemier's, cancer, major depression and inflammatory bowel disease (IBD).

TNF can bind to two receptors such as TNF-R1 and TNF- R2. TNF-R1 found in most tissues whereas TNF-R2 found only in immune cells. In its transducer mechanism three pathways are activated such as, a) Activation of NF-κ B, cell survival and proliferation, anti-apoptotic factors are mediated, b) Activation of the MAPK pathways, c) activation of c-Jun N- terminal kinases (JNK) pathways which involved in cell proliferation and pro apoptotic.

3.5.5 Over expression of anti-apoptotic gene

Bcl-2 (B cell lymphoma 2), and Bcl- XL are belong to the family of Bcl-2. Those are encoded by Bcl-2 gene. It plays a role in regulating apoptosis. The Bcl-2 gene has been involved in a number of cancers including melanoma, breast, prostate, chronic lymphocytic leukemia and lung carcinomas. It is also involves in conventional chemotherapy resistance.

In lymphoma, mutation at fourteenth and eighteenth place of chromosome in heavy chain of immunoglobulin, where the gene Bcl-2 fused, leading to transcription of excessive levels of Bcl-2. This deceases the propensity of these cells for undergoing apoptosis.

3.5.6 Expression of oxygen regulated protein

The 150-kd oxygen- regulated protein **(ORP150)** also called as hypoxia up- regulated protein 1 encoded by HYOUI gene. The protein encoded by this gene is thought to play an important role in protein folding and secretion in the ER. Since suppression of the protein is associated with accelerated apoptosis, it is also have an important cyto-protective role in tumors, especially in breast tumors, and thus it is associated with tumor invasiveness. When the subjected to the tumor environmental stress such as oxygen deprivation, it redirects biosynthetic pathway to express 150Kda protein to maintain the cell viability.[25]

3.5.7 Role of interleukins

Interleukins are group of cytokines, produced by wide variety of body cells. Among family of interleukins, some plays a major role in breast cancer.

Interleukin 6 (IL-6) is an interleukin that acts as both a pro- inflammatory and anti-inflammatory cytokine. In humans, it is encoded by the IL6 gene. Interleukin-6 (IL-6) plays a primary role in breast cancer pathophysiology. When cancer cells exposed to IL-6 the resistance towards drug is increased. Up regulation of it cause the cells to metastasize to bone. Advanced or metastatic cancer patients have higher levels of IL-6 IN their blood. Hence there is an interest in developing anti-IL-6 agents as therapy against several of these diseases.

Interleukin 4(IL-4), is a cytokine that induces differentiation of native helper T cells to Th2 cells. Upon activation by IL-4, Th-2 cells subsequently produce additional IL-4. It stimulates the activated B- cell and T- cell proliferation, and the differentiation B cells into Plasma Cells, and also induces B- cell class switching to IgE, and up-regulates MHC class II production. IL-4 decreases the production of Th1 cells, macrophages, IFN-gamma, and dendritic cell IL-12.[26, 27]

3.6 BREAST CANCER STAGING AND TREATMENT

3.6.1 STAGING

A staging system is used to classify the extent of disease based on the tumor size, location (e.g. ducts, lobules), involvement of lymph nodes and has spread to surrounding tissue (e.g. chest wall, skin of breast) or distant organs (e.g. lungs, liver, brain, bone).[20]

3.6.2 TREATMENT [28]

Based upon tumor size, location, involvement of lymph nodes and metastasis, risk and stages of the disease, appropriate treatment will be given.

1. **SURGERY** (based on biopsy)**:** It includes,

   Lumpectomy or breast – conserving surgery

   - Removal of lump and surrounding tissues

Mastectomy

-Removal of all the breast tissues

Preventive surgery,

-Prophylactic mastectomy for women at risk and prophylactic ovary removal to lower estrogen production in the body.

2. **RADIATION THERAPY**

It is used to destroy the remaining cancerous cells left behind after surgery.

- **External Beam Radiation,** which is well tested, long lasting treatment option,
- **Internal Beam Radiation** (implantation of radioactive seeds), which is recently developed, shorter treatment interval, focused to the affected area so low exposure but fewer adverse effects.

3. CHEMOTHERAPY.

It is a systemic therapy, used to either to shrink the tumor or to reduce the risk recurrence. For advantage- stage disease, it is destroy as several cancer as possible. These are the chemotherapy drugs approved by FDA for breast cancer,

Alkylating agent:

Nitrogen mustard	-	Cyclophosphamide

Antimetabolites:

Folate antagonists	-	Methotrexate
Pyrimidine antagonists	-	5-Flurouracil
Taxanes	-	Paclitaxel, Docetaxel
Antibiotics	-	Doxorubicin
Others	-	Capecitabine, Everolimus

4. HORMONAL THERAPY

It is given for the patients with hormone receptor-positive cancers. It is used to reduce the amount of estrogen or block its action to reduce the risk of recurrence at the early stage of the disease and to shrink or slow down the growth of existing tumor at the advanced stage of the disease.

Aromatase inhibitors	-	Latrazole, Anastrazole, Exemestane

Selective estrogen

Receptor modulators	-	Tamoxifen, Tormifene

Estrogen receptor

Down regulators	-	Fulvestran

3.7 DRUG PROFILE OF KANCHANARA GUGGULU VATI

(Polyherbal formulation)

3.7.1 COMPOSITION OF KANCHANARA GUGGULU VATI [29]

Kanchnar guggulu vati is an ayurvedic polyherbal formulation, consisting of 12 medicinal plants.

Table 1: Composition of ingredient(s) present in poly herbal formulation, Kanchanara guggulu vati

Botanical name	Parts used	Quantity (%)
Bauhinia variegata	Stem bark	24.10%
Terminalia chebula	Fruit	4.82%
Terminalia bellerica	Fruit	4.82%
Emblica officinalis	Fruit	4.82%
Zingifer officinale	Rhizome	2.41%
Piper longum	Dried fruit	2.41%
Piper nigrum	Dried fruit	2.41%
Crataeva nurvala	Stem bark	2.41%
Elettaria cardamomum	Dried fruit	0.60%
Cinnamomum zeylanicum	Stem bark	0.60%
Cinnamomum tamala	Leaf	0.60%
Commiphora weightii	Oleo resin	50%

3.7.2 PROFILE OF INDIVIDUAL INGREDIENTS OF KANCHANARA GUGGULU VATI

1. ***BAUHINIA VARIEGATA***

BOTANICAL NAME : *Bauhinia variegata*

FAMILY : Ceasalpiniaceae

PARTS USED : Stem bark

DISTRIBUTION : distributed throughout India, except Jammu Kashmir, Himachal Pradesh, Sikkim

VERNACULAR NAMES

Tamil : Sihappumandarai

English : Mountain Ebony

Hindi : Kachanaar, orhid tree

Telugu : Bodanta

Malayalam : Chovanna, mandaru

PHARMACOLOGICAL ACTIVITY

- The bark, flowers and root are used as a cataplasm
- Root decoction used in dyspepsia
- Flowers used as laxative
- And bark used as tonic and anthelmintic

2. *TERMINALIA CHEBULA*

BOTANICAL NAME	:	*Terminalia chebula*
FAMILY	:	Combretaceae
PARTS USED	:	Fruit
DISTRIBUTION	:	Distributed throughout Southeast Asia- India, Southeast Asia- India, Srilanka, Bhutan, Nepal, Malaysia

VERNACULAR NAME

Tamil	:	Kadukaay
English	:	Black or chebulicmerobalan
Hindi	:	Haritaki
Telugu	:	Nallakaraka
Malayalam	:	Katukka

PHARMACOLOGICAL ACTIVITY

- Astringent
- Bleeding condition
- Anti-bacterial
- Anti-ulcer
- Anthelmintic
- Laxative
- Stomachic and tonic

3. *TERMINALIA BELLERICA*

BOTANICAL NAME Terminalia bellerica

FAMILY : Combretaceae

PARTS USED : Fruit

DISTRIBUTION & HABITAT : Distributed throughout Indian Forests and plains.

VERNACULAR NAME

Tamil : Tanri

English : Beach almond

Hindi : Bahuvirya

Telugu : Tandra

Malayalam : Thaanni

PHARMACOLOGICAL ACTIVITY

- The dried fruits used as astringent, tonic and laxative
- Also used in piles, diarrhoea
- Bark of the plant is used as to increase diuresis

4. *EMBLICA OFFICINALIS*

BOTANICAL NAME : Emblica officinalis

FAMILY : Phyllanthaceae

PARTS USED : Fruit

DISTRIBUTION : Found throughout India, the sea coast districts and on hill Slopes upto 200 meters also cultivated in plains.

VERNACULAR NAMES

Tamil	:	Nelli
English	:	Amla, Indian gooseberry
Hindi	:	Aonla
Telugu	:	Usiri
Malayalam	:	Nelli

PHARAMACOLOGICAL ACTIVITY

- Fresh fruit is refrigerant, tonic, antiscorbutic, diuretic and laxative, blood purifier, diarrhea and jaundice
- Syrup from the fruit is antibilous, diuretic and cooling; used in fever, vomiting, indigestion and habitual constipation
- The root bark is used in aphthous stomatitis
- The root is used in jaundice treatment, dyspepsia, and cough
- The shoots are used in diarrhea, indigestion

5. *ZINGIBER OFFICINALE*

BOTANICAL NAME	:	Zingiber officinale
FAMILY	:	Zingiberaceae
PARTS USED	:	Rhizome
DISTRIBUTION	:	Distributed in China, India, Indonesia, Nepal, Nigeria, and Thailand

VERNACULAR NAME

Tamil	:	Inji
English	:	Ginger
Hindi	:	Adrak
Telugu	:	Allam
Malayalam	:	Inchi

PHARMACOLOGICAL ACTIVITY

- The rhizome is used as a stimulant, carminative
- It is used in dyspepsia, vomiting, rheumatism, dysentery, cholera, piles
- Also promotes menses in amenorrhoea

6. *PIPER LONGUM*

BOTANICAL NAME	:	Piper longum
FAMILY	:	Piperaceae
PARTS USED	:	Dried fruit
DISTRIBUTION	:	Distributed in shady floors and village grove in India Found in Kerala, globally found in Indonesia, Madagaskar

VERNACULAR NAME

Tamil	:	Tippili
English	:	Long pepper
Hindi	:	Pippal

PHARMACOLOGICAL ACTIVITY

- Thermogenic, tonic
- Diuretic, purgative
- Expectorant, anorexia
- Splenomegaly, aphrodisiac
- Fever, digestive, and general debility
- Bronchitis
- Disease of spleen
- Tumors

7. *PIPER NIGRUM*

BOTANICAL NAME	:	Piper nigrum
FAMILY	:	Piperaceae
PARTS USED	:	Dried fruit
DISTRIBUTION	:	Native for black pepper is Malabar region of Southwestern India. Now it is grown in various tropical regions, including India, Indonesia and Brazil

VERNACULAR NAMES

Tamil	:	Karumilagu
English	:	Black pepper
Hindi	:	Mirch
Telugu	:	Miryalatige
Malayalam	:	Karumulaku

PHARMACOLOGICAL ACTIVITY

- Dried unripe fruits are stimulant, carminative and stomachic
- Also used as diuretic, and anti-diabetic agents

8. *CRATAEVA NURVALA*

BOTANICAL NAME	:	Crataeva nurvala
FAMILY	:	Capparidaceae
PARTS USED	:	Stem bark
DISTRIBUTION	:	It is widespread from India to Southeast Asia, South of China

VERNACULAR NAME

Tamil	:	Maralingam
English	:	Three hand capu
Hindi	:	Baruna
Telugu	:	Bilvarani
Malayalam	:	Neermatalam

PHARMACOLOGICAL ACTIVITY

- Anticancer activity
- Anti-urolithiasis
- Migraine
- Intestinal worms

9. *ELETTARIA CARDAMOMUM*

BOTANICAL NAME	:	Elettaria cardamomum
FAMILY	:	Zingiberaceae
PARTS USED	:	Dried fruit
DISTRIBUTION	:	Distributed in evergreen monsoon forests of the western Ghatz in Southern India and western highland in Srilanka

VERNACULAR NAMES

Tamil	:	Elam, Elakkaai
English	:	Cardamom
Hindi	:	Chhoti Elachi
Telugu	:	Yelakayalu
Malayalam	:	Elakkaya, Citalam

PHARMACOLOGICAL ACTIVITY

- Eases stomach pain
- Carminative, aromatic
- Anti-spasmodic

10. *CINNAMOMUM ZEYLANICUM*

BOTANICAL NAME	:	Cinnamomum zeylanicum
FAMILY	:	Lauraceae
PARTS USED	:	Stem bark
DISTRIBUTION	:	Native is Indian subcontinent, Nebal, Bhutan, Pakistan but most specifically in Sri Lanka. Also found in Brazil, Madagaskar, Vietnam

VERNACULAR NAMES

Tamil	:	Channalavangam
English		Cinnamon
Hindi	:	Dalchini
Telugu	:	Dasini
Malayalam	:	Elavangam

PHARMACOLOGICAL ACTIVITY

- Warming stimulant, carminative
- Antispasmodic, anti-septic
- Anti-viral

11. *CINNAMOMUM TAMALA*

BOTANICAL NAME	:	Cinnamomum tamala
FAMILY	:	Lauraceae
PARTS USED	:	Leaf
DISTRIBUTION	:	Native is India, Nepalese cinnamon is Popular for its medicinal properties

VERNACULAR NAMES

Tamil	:	Talishapattiri
English	:	Indian bay leaf
Hindi	:	Tejpatta
Telugu	:	Tasilapatri
Malayalam	:	Tamalapatram

PHARMACOLOGICAL ACTIVITY

- The leaves are used in the treatment of colic and diarrhea
- Hypoglycemic activity
- Anti-fungal activity

12. *COMMIPHORA WEIGHTII*

BOTANICAL NAME	:	Commiphora weightii
FAMILY	:	Burseraceae
PARTS USED	:	Exudate
DISTRIBUTION	:	Guggul is native to India, Pakistan and Arabia in India it is found in Rajastan, Gujarat, Madhya Pradesh and Karnataka

VERANACULAR NAME

Tamil	:	Mahisaksiguggalu
English	:	Indian badellium
Hindi	:	Guggulu
Telugu	:	Guggipannu
Malayalam	:	Gulgulu

PHARMACOLOGICAL ACTIVITY

- Anti-bacterial
- Paralysis
- Rheumatoid arthritis
- Gout
- Anti-inflammatory
- Anti-viral
- Anti-fertility
- Anti-obesity

3.8 PHARMACOLOGICAL STUDIES

3.8.1 BAUHINIA VARIAGATA

Amita Mishra et al. (2013), assessed the antibacterial, antioxidant, and anticancer activity of various leaf extracts of *B. variagata.* Acetone fraction exhibited appreciable reducing power at all the test concentration. Benzene, ethyl acetate, and water extracts were similar with minor differences. Water fraction was found to be the most active exhibiting percentage growth inhibition against prostrate, lungs (hop-62), ovary, Breast (mcf-7), cell lines respectively.

Ethyl acetate extract showed marked cytotoxicity against mcf-7(84%), thp-(93%) cell lines. In general, breast (mcf-7) leukemia (thp-1) cell lines exhibited greater sensitivity to *Bauhinia variegate* extracts. [30]

Sonam Pandey et al. (2012), evaluated the antioxidant and free radical scavenging activity of *Bauhinia variagata* by *in-vitro* methods. Among the various extracts methanol extracts showed good antioxidant activity. IC_{50} value of *B. variagata* leaf, stem bark and floral buds are 17.9, 19.5 and 17.2 µg/ml respectively.[31]

M.M.Ghiaisas, et al. (2008) evaluated the immuno modulatory activity of ethanolic extracts of the stem bark of *B .variegata* using swice albino mice. On oral administration, extract showed a significant increase in the primary and secondary humoral antibody responses, by increasing the hemagglutinating antibody titre at doses of 250 and 500 mg/kg there was a significant increase in the phagocytic index and percentage neutrophil adhesion at doses of 250 and 500 mg/kg. This study reveals that the *B. variagata* holds a promise as an immune modulatory agent, which acts by stimulating both the specific and non-specific arms of immunity. [32]

H. Bodakhe, et al, (2007), assessed the hepato protective properties of bark extract of *Bauhinia variagata* in CCl4 induced cirrhosis in male Sprague dawly rats. Stem bark extracts at 200 mg/kg dose showed better anti-hepatotoxic activity. Hence, *B.variegata* appears to be a promising hepatoprotective agent. [33]

SM. Bairagi et al. (2012), evaluated the anti-inflammatory activity of methanol and aqueous fraction of the bark of *B.variegata* by dextran induced edema method in albino rats. Anti-inflammatory activity determined by carrageenan induced paw edema and dextran induced paw edema. The anti-inflammatory activity determined by carrageenan induced paw edema were not too significantly different (P>0.05) from the control. Significant activity against dextran induced paw edema in rats was exhibited by both methanol extract (P<0.01) and aqueous extract (P<0.05) when administered orally at 200 mg/kg. [34]

3.8.2 TERMINALIA CHEBULA

Kannan.P et al. (2009), evaluated the anti-bacterial activity of ethanolic fruit extract of *T. chebula* against clinically important standard reference bacterial strains. The fruit extract was highly effective against Salmonella typhi and the MIC was determined as 1mg/ml.[35]

Rohini Ahuja et al. (2013), investigated the *invivo* and *invitro* anticancer activity of *T. chebula* fruits against ehrlich ascites cells in swiss mice. The high dose of ethanolic extract of *T. chebula* (200mg/kg, orally) significantly reduces the tumour growth which was demonstrated by increased lifespan of the mice and restoration of hematological parameters.

ETC was also found to be cytotoxic *in-vitro* parameter which shows that ETC possesses significant anticancer potential. *T. chebula* against buffalo rat liver 3A, MCF-7, A -

549 cell lines. The extracts was potent 1.7µg/ml, 643.13± 4.2µg/ml, 208.16± 3.7µg/ml, respectively.

The extract showed the higher content of total phenolic and flavonoids was directly associated to higher cytotoxicity activity. The ethanolic leaf gall extracts of *T. chebula* showed effective cytotoxicity activity, which might be attributed to the phenolic / flavonoids present in higher concentration.[36]

Koteswara Rao et al. (2006), investigated the chloroform extract of *T. chebula* seed powder for its anti-diabetic activity in STZ induced diabetic rats using short term and long term study protocols. The efficacy of the extract was also evaluated for the protection of renal functions in diabetic rats. The chloroform extract of *T. chebula* seeds produced dose dependent reduction in blood glucose of diabetic rats when compared with the standard drug (Glibenclamide).

In short term study the maximum reduction in blood glucose of 20.85%, ($p<0.01$), 28.45% ($p<0.001$) and 42.20% ($p<0.001$) at four hours with doses of 100,200,300mg/kg respectively. In long term study (4 weeks) also it produce significant reduction in blood glucose. The maximum reduction in blood glucose of 53.09% ($p<0.01$), when compared with control glibenclamide 60.10% ($p<0.01$).[37]

Sarmistasaha et al. (2014), investigated the antioxidant activity of poly phenolic extract of *T. chebula* by various *in vitro* methods. The antioxidant activity of the extract is significantly higher than the standard ascorbic acid, and its activity is concentration dependent.[38]

3.8.3 TERMINALIA BELLERICA

Amal M.Saad et al. (2014), evaluated the antioxidant and cytotoxic effect of *T. bellerica* against HepG-2 cell line. The reducing power was ranged from (2.75 at 200µg/ml), with respect to the ascorbic acid (2.55). On the other hand, the results of HepG-2 assay showed that extract have cytotoxic activity with IC50 of 19.35µg/ml.[39]

Sharangouda J. Patil et al. (2010), evaluated the effect of *T. bellerica* bark various extracts on activities of accessory reproductive duct in male rats. Adult male rats were administered with 10mg, 25mg/100g of ethanolic and benzene extracts for 50 days showed decreased in the weight of accessory reproductive ducts and epididymal sperm count were significantly decreased. In conclusion, out of two extracts tested, ethanolic extract at the dose of level of 25mg/100g body weight is having more anti-spermatogenic and anti-steroidogenic activities in male rat reproductive system.[40]

M.C Sabu et al. (2008), evaluated the effect of continuous administration of methanolic extract of fruits of *T. bellerica* in alloxan induced hyperglycemia and antioxidant mechanism in rats. *T. bellerica* prevented alloxan induced hyperglycemia from the 6^{th} day of administration and there was 54% reduction on 12^{th} day ($p<0.001$). Similarly there was significant increase in the activity of catalase in blood and liver. These results suggested that *T. bellerica* fruit extract possessed anti-diabetic and anti-oxidant activity and these activities may be interrelated.[41]

RenuKadian et al. (2014), evaluated the antipsychotic potential of *T. bellerica* in experimental animal models. The fruit powder administered the concentration of (4%w/w, 6%w/w, 8%w/w) for 15 consecutive days. Further, the biochemical estimation were done by

estimating brain dopamine levels. And it showed TBFP significantly decreased the dopamine levels. The results suggest that TBFP possesses anti-psychotic activity.[42]

3.8.4 EMBLICA OFFICINALIS

SatishK.verma et al. (2012), evaluated the cytotoxic activity of *Emblica officinalis* whole plant extract against various cell lines namely of lung cancer (A-549), liver (Hep-2), colon (502713HT-29) and neuroblastoma (IMR-32). The activity was done using 100µg/ml of the extract against lung (A-549) cell line showed 82% of growth inhibition. In case of liver (Hep-2) showed no activity reported, where as in case of colon 502713 cell line plant extract showed maximum activity and for neuroblastoma (IMR-32) Cell line showed 97% growth inhibition.[43]

Gupta priya et al. (2012), evaluated the antimicrobial and antioxidant activity of seed extract of *E. officinalis* and the study concludes that seed extract contain high anti-bacterial and antioxidant property.[44]

MahaveerGolechha et al. (2014), investigated the anti- inflammatory activity of hydroalcoholic extract of *E. officinalis* in rodent models of acute and chronic inflammation. The extract was administered at the dose of 300,500,700mg/kg. At the dose of 700mg/kg the extract shows maximum anti-inflammatory activity in all experimental models, and the effects were comparable to that of the standard anti-inflammatory drugs.[45]

Bheemshetty et al. (2015), evaluated the effect of ethanolic extract of *E.officinalis* on histopathology of kidney and on biochemical parameters in hyperlipidemic albino wistar rats. The ethanolic extract administered as the dose of 100mg/kg body weight daily, and the test dose treated hyperlipidemic animal model expressed changes in renal markers, biochemical parameters and histopathology of kidney.[46]

3.8.5 ZINGIBER OFFICINALE

Nalbantsoy et al. (2008), evaluated the antimicrobial and cytotoxic activities of ethanol and chloroform extracts of *Z. officinale.* The cytotoxic activity investigated against Hela (Human Cervical Cancer) and mouse fibroblast (L929) cell lines. The antibacterial activity was tested by the paper disc diffusion technique. IC50 values versus mouse fibroblast and Hela cells were found to be 7.28µg/ml and 74.32µg/ml, respectively, for the chloroform extract, while the ethanol extract showed IC50 values at 101µg/ml and 33.78µg/ml, respectively.

The antimicrobial activity results showed that the ginger extracts inhibited the growth of 5 out of 8 microorganisms but had no effect on the growth of E.coli, Pseudomonas aeroginosa and Stephylococcus epidermidis.[47]

Silvia Mosovska et al. (2015), evaluated the antioxidant activity of ginger extract. A method for the extract was chosen, which enabled to isolate mostly phenolic compounds (181.41mg/g of extract) from which flavonoids contributed to 7.8% (14.15mg/g of extract). NMR analysis showed that the phenolic ketones were predominant in the extracts of whole phenolic compounds. According to results of two spectrophotometric methods (ABTS& DPPH test), ginger extract showed stronger ability to scavange DPPH radical than ABTS cation radical.[48]

ShafinaHanimmohd Habib et al. (2005), evaluated the anticancer and anti-inflammatory effects of ginger extracts on Ethaionine induced hepatoma in rats. Ginger extracts significantly reduced the elevated expression of NFκb through the suppression of the pro-inflammatory TNF-α.[49]

P.Paul et al. (2012), evaluated the hypolipidemic effect of ginger extract in vanaspati fed rats. The extract consequently administered (300mg/100ml/g. body weight/day) orally for 49 day. Simultaneous administration of ginger extract significantly ($p<0.05$) prevented the rise in total cholesterol, LDL, triglyceride level and rise HDL. In histo pathological study, no significant changes were found in the liver and aorta of all the treated groups as compared with control group. It is concluded that ginger extract showed hypolipidemic effect in vanaspati fed rats.[50]

S.S Prasad et al. (2012), investigated the action of ginger-juice on blood coagulation process in rat. Chronic administration of ginger- juice (2ml& 4ml. p.o for 30 days) caused an increase in the bleeding time.[51]

3.8.6 PIPER LONGUM

Beena Joy. (2010), studied the antioxidant, anticancer, and antimicrobial activity of various fruit extracts of *Piper longum.* For antioxidant activity, out of the different extracts tested, the ethyl acetate extract and cold hexane: water extracts (1:1) showed better antioxidant activity. The screening of antimicrobial and antitumor effects of *Piper longum* showed the better efficacy of those two extracts. The antitumor studies showed a dose dependent behavior of the extracts towards leukemic cell lines K562. Among the three bacteria tested, hot ethyl acetate extract showed more potency against E.coli than Bacillus subtitlis and was found to be less active for staphylococcus aurus. The antioxidant, antitumor, and anti-microbial activity found to be higher for hot extracts than cold extracts.[52]

Archana.D et al. (2015), evaluated the antioxidant anti-clastogenic potential of methanolic extract of *P.longum.* The GC-MS analysis provides different peaks determining the presence of eighteen phytochemical compounds with different therapeutic activities. The

methanol extract at a concentration of 40µg/ml showed the highest antioxidant activity by DPPH assay (64.42%) comparable to standard, ascorbic acid (73.68%). The reducing power observed was in the order of 40µg/ml>20µg/ml>10µg/ml.

The production afforded by extract against the cytotoxicity of peripheral blood lymphocytes were confirmed by micronucleus (MN) assay and the methanolic extract treatment decreased the frequency of MN in a concentration dependent manner.[53]

Alok S Wakade et al. (2008), investigated the effect of methanolic extract of fruits of *P.longum* on the biochemical changes, tissue peroxidase damage and abnormal antioxidant levels in Adriamycin induced cardio toxicity in Wistar rats. Chronic oral administration of methanolic fruit extract 250mg/g and 500mg/kg prevented Adriamycin induced alterations in marker enzyme activity, endogenous antioxidant levels and cellular damage ($p<0.05$).[54]

Shaik Abdul Nabi et al. (2013), evaluated the antidiabetic and antihyperlipidemic activity of *P.longum* root extracts in STZ induced diabetic rats. Among the different extracts were tested for their glucose lowering effects, the methanolic and aqueous extracts at a dosage of 200mg/kg produced maximum fall of 39% and 75% respectively, in the FBG levels of diabetic rats after 6 h of treatment. The administration of same dose for 30 days in diabetic rat resulted in a significant decrease in FBG levels in the corrections of diabetic dyslipidemia compared to untreated diabetic rats ($p<0.1$).[55]

3.8.7 PIPER NIGRUM

Somchai Sriwiriyajan et al. (2015), investigated the anticancer and cancer prevention effects of piperine- free *P.nigrum* extract against breast cancer cell line and N-nitrosomethylurea induced mammary tumorigenesis in rats. The extract was a highly effective against the MCF-7 with IC50 value of 7.45µg/ml and showed lower values for the

other cell lines. And the tumor size of the treated group extract at the dose of 100, 200 and 400mg/kg was significantly reduced when compared with the control and vehicle groups. [56]

FarhanaTasleem et al. (2014), evaluated the analgesic and anti-inflammatory activities of hexane and ethanol extract of *P.nigrum* fruit in mice and rats. Piperine at a dose of 5mg/kg and ethanol extract at a dose of 15mg/kg after 120 min and hexane extract at a dose of 10mg/kg after 60 min exhibited significant ($P<0.05$) analgesic activity. In the evaluation of anti-inflammatory effect, piperine at a dose of 10 and 15mg/kg started producing anti-inflammatory effect after 30 min, which lasted till 60 min, whereas hexane and ethanol extract also produced a similar activity at a slightly low dose (10mg/kg) but lasted for 120 min. [57]

Amin F. Majhalawieh et al. (2009), investigated the immunomodulatory and anticancer effect of *P.nigrum* and cardamom by *in-vitro* methods. The results showed that the black pepper and cardamom aqueous extracts significantly enhance splenocyte proliferation sin a dose dependent, synergistic fashion ($P<0.01$). Experimental evidence suggest that the extracts exert pro- inflammatory and anti- inflammatory roles, and it significantly enhance the cytotoxic activity of natural killer cell, indicating their potential anti-cancer effects.[58]

Md. Gayasuddin et al. (2013), investigated the effect of ethanolic extract of *P.nigrum* fruits on midazolam induced hypnosis in rats. Study result showed that sub chronic treatment of 100mg/kg of extract p.o for five consecutive days on hypnotic dose (50mg/kg) of midazolam in rats significantly increased the sleeping time in pretreated group to 35% to that of control value without affecting the sleeping latency.[59]

3.8.8 CRATAEVA NURVALA

Swati N.Hade et al. (2016), evaluated the antioxidant, anti-proteolytic, and cytotoxic effects of stem bark extract of *C.nurvala* against hepato-carcinoma and mouse melanoma cell lines. Among different extract the methanolic extract showed better radical scavenging activity. The extract showed good proteolytic activity with 26.04% inhibition on BSA and the IC50 values stem bark extract against HepG2 and B16F0 cells using MTT were determined to be 34.67µg/ml, and 49.43µg/ml after 48 h, respectively. [60]

AtanuBhattacharjee et al. (2014), investigated the antioxidant and anti-demantia activities of *Crataeva nurvala* stem bark extract against scopolamine induced cognitive impairment via antioxidant activities in rats. Sub-acute exposures (daily, for 7 successive days) of stem extract (100, 200, and 400 mg/kg) significantly increased antioxidant enzyme activities (SOD and CAT), total content of reduced GSH and reduced lipid peroxidation (MDA level) in rat brain homogenates in dose dependent manner, suggesting the antioxidant potential. Moreover, the Hebb's William maze test, the extract showed significant improvement in transfer latency, suggesting anti-demantia activity.[61]

Bhaskar et al. (2009), evaluated the anti-fertility activity of stem bark extract of *C. nurvala* in rats. The ethanol and aqueous extracts exhibited partial and complete resorption of implants at 300 and 600 mg/kg dose levels, respectively. In estrogenic activity study, both the extracts increased uterine weight and caused opening and cornification of vagina in immature rats. The present study justifies its effectiveness in preventing pregnancy in all rats at dose level of 300 and 600mg/kg.[62]

SikarwarMukesh S et al. (2012), investigated the antihyperlipidemic activity of *Crataeva nurvala* stem bark extracts in triton and atherogenic diet-induced heperlipidemic rat

model. Among various extracts, oral administration of 500 mg/kg of the ethanolic extract and aqueous extract exhibited reduction (p0.01) in serum lipid parameters like total cholesterol, triglycerides, LDL, VLDL, and increase in HDL in hyperlipidemic rats in comparison with hyperlipidemic control in both model. [63]

3.8.9 ELETTARIA CARDAMOMUM

Samir Qiblawi et al. (2012), evaluated the chemo preventive effect of cardamom on chemically induced skin carcinogenesis in swiss albino mice. A significant reduction in the values of tumor incidence, tumor burden, and tumor yield of papillomas was observed in mice treated orally with 0.5 mg of cardamom powder in suspension continuously at pre-, peri-, post-initiational stages of papillomagenesis compared with the control group. Treatment of cardamom suspension by oral gavage for 15 days resulted in a significant decrease in the lipid peroxidation level of the liver ($P<0.1$).

In addition, the reduced glutathione level was significantly elevated in comparison with the control group ($P<0.5$) following cardamom suspension treatment. Taken together, these findings indicate the potential of cardamom as a chemo preventive agent against two-stage skin cancer.[64]

SweetyKumari et al. (2013), studied the protective effect of *E. cardamomom* against pan masala induced damage in lung of male swiss mice. Lung of pan masala treated group showed adenocarcinoma, edema, and inflammation with increased activity of acid phosphatase, alkaline phosphatase, and lactate dehydrogenase. The deleterious effects were seen to be less in cardamom treated group and the enzymatic activity also decreased significantly ($P<0.05$) in the ameliorating group.[65]

BheenaKhillare et al. (2014), evaluated the spermicidal activity of cardamom and cumin seed extracts in albino rats. Under the test conditions, minimum effective concentrations for cardamom and cumin seeds extract are 1mg/million sperm and 5mg/million sperm respectively. The sperm was also studied for their morphology and viability of sperm was also studied and no change was observed in morphology of head, mid-piece and tail and no viable sperm seen. [66]

Jamal.A et al. (2005), investigated the anti-ulcerogenic activity of *Elletaria cardamommum* and *Amomum subulatum* in albino rats. Results obtained from experimental model of ethanol induced acute ulcer in rats showed 76.36% protection when 50mg/kg essential oils of cardamom, in case of oils of A. subulatum it was 60.91% inhibition. Thus, it is concluded that cardamom is more potent in inhibiting gastric ulcer than A. subulatum.[67]

3.8.10. CINNAMOMUM ZEYLANICUM

B.Varalaxmi et al. (2013), evaluated the antimicrobial and anticancer activities of *C.zeylanicum* bark extracts against Hep G2 cell line. Among the various extract the methanolic extract showed an IC50 value of 150µg/ml against HepG2 MTT assay. In antimicrobial results also showed the methanolic extract had better antibacterial and antifungal activity. The extract showed MIC value of 2.5mg/ml for *Bacillus subtilis* and 5mg/ml for *Asper*gillus niger. [68]

UlkuGulchihanSimsek et al. (2013), evaluated the antioxidant activity of cinnamon bark oil (*Cinnamon zeylanicum* L.) in Japanese quails under thermo neutral and heat stressed conditions. Cinnamon oil supplementation to diet significantly increased antioxidant enzyme activity and GSH level of the tissues in both environmental conditions ($P<0.01$). Dose of 500 ppm cinnamon oil had strong effect on antioxidant activity of the internal organs ($P<0.01$). In

conclusion, cinnamon oil supplementation to diet reduced the adverse effects of heat stress and resulted the protective effect on the internal organs by activating antioxidant mechanism. [69]

PaulinNyadjeu et al. (2011), evaluated the antihypertensive and vasorelaxant effects *C. zeylanicum* stem bark aqueous extract in rats. The extract at a dose of 5, 10, 20 mg/kg induced a significant reduction in mean blood pressure in salt-loaded hypertensive, and spontaneously hypertensive rats in dose dependent manner.[70]

Kalpana Joshi et al. (2009), investigated the anti-inflammatory activity of *C. zeylanicum* by in-vitro and in- vivo methods. Both the methods were targeting TNF-α using flow cytometry. Ethanol extract showed suppression of intracellular release of TNF-α in murine neutrophils as well as leukocytes in plural fluid. The extract was found to be inhibit TNF-α gene expression in LPS- stimulated human PBMCs at 20µg/ml concentration. [71]

3.8.11. CINNAMOMUM TAMALA

DurreShahwar et al. (2015), evaluated the anticancer activity of *C. tamala* towards human ovarian cancer cells. The leaf extract were isolated through spectroscopic method, among four isolated compounds, no: 1 exhibited highest cytotoxicity with 90.16±1.06% inhibition followed by compound 2 with 84.40±1.53% inhibition, while compounds 3 and 4 were inactive in the bioassay.[72]

S Lashmi Devi et al. (2007), evaluated the antioxidant activity of *C. tamala* using brain synaptosomes of diabetic rats as model system. Methanolic leaf extracts contains 6.7mg gallic acid /100g, and it displayed scavenging activity against superoxide and hydroxyl radicals in a concentration dependent manner. Maximum inhibition of lipid peroxidation, scavenging activity, and reducing power of leaf extract were observed at a concentration of

220µg. These effects of leaf extract were comparable with that of BHT a synthetic antioxidant.[73]

RupeshSoni et al. (2013), evaluated the effect of ethanolic extract of *C. tamala* leaves on wound healing in STZ induced diabetic rats. In the excision wound model the wound area and day of epithelization both were significantly decreased in ethanolic extract treated rats. In incision wound model the significantly higher tensile strength was observed in rats treated orally (100mg/kg) with ethanolic extract. The results suggested that the ethanolic leaf extract can be used in treatment of delayed wound healing in diabetic rats.[74]

Manoj K Dalai et al. (2013), investigated the acetyl cholinesterase (AchE) and butyl cholinesterase (BchE) inhibitory activity of the extract of *C. tamala*. The outcome of the study demonstrated that the cinnamon oil obtained from the leaves of *C.tamala* possesses maximum inhibition against AchE (IC50= 94.54µg/ml) and BchE (IC50=135µg/ml). Hence, this plant may be explored as anti-cholinesterase agent further for the better and safer management of Alzhemier's diseases.[75]

3.8.12COMMIPHORA WIGHTII

Michelle xiao et al. (2012), revealed that Guggulu is having multi targeted chemo preventive and chemotherapeutic property by a review article. This study showed that guggulu is mainly active against prostate cancer cell line because it contains number of steroids, including two isomers Z-and E-guggulusterone. The IC_{50} 1µmol/L against PC-3 cell line. The guggulu mediated suppression of cancer cell proliferation has also been reported in human breast cancer cells, head and neck cancer cells, leukemia cells, lung cancer cells, skin cancer cells, and colon cancer cells.[76]

Qureshi Ikram et al. (2014), evaluated the antioxidant activity of *Commiphora wightii* by various methods like anti-lipid peroxidation assay, metal chelating activity, DNA damage inhibition. In all these method guggulu extract exhibit good scavenging activity with the IC_{50} value=934µg/ml.[77]

Monika singh et al. (2013), investigated the antifungal activity of ethanolic extract *C.wightii* against various pathogenic fungi. The results showed that there was an increase in concentrations the rate of growth inhibition also increases. Further shows that the growth of these fungi inhibits more in presence of higher concentrations as compared to lower concentrations of extract.[78]

Sumitkumar et al. (2016), evaluated the anti-ulcer activity of aqueous extract of *C.*wightii by using indomethacin and ethanol induced gastric model in rats. Percentage ulcer inhibition of aqueous extract at 500mg/kg for indomethacin and ethanol induced ulcer model were 23.27%, 40.55% respectively, when comparing with standard drugs ranitidine and misoprostol.[79]

3.9. REFERENCE FOR METHODOLOGY

3.9.1. PHYTOCHEMICAL ANALYSIS

Prathima Shetty et al. (2013), assessed the preliminary phytochemical screenig of ethanolic extract of *Elettaria cardamomum* fruits. In the study, the ethanolic extract showed the presence of alkaloids, saponins, flavonoids, tannins and phenolic compounds, terpinoids, and phytosterols, fixed oils, carbohydrates and protein.[80]

Veena Sharma et al. (2013), assessed the phytochemical investigation of leaves and stems of *Achyranthus aspera* Linn. Results revealed that both stem and leaves possesses the phytochemicals like alkaloids, terpenoids, flavonoids, cardiac- glycosides, saponins in different amount.[81]

3.9.2ANTIOXIDANT ACTIVITY

M. Ragavendra et al. (2013), investigated the antioxidant activity of methanolic leafy extracts from six edible leafy vegetables. The methanolic extracts were studied for antioxidant activity by various method including DPPH method by using ascorbic acid as standard.[82]

Y. Lu et al. (2014), determined the antioxidant activity of citronellal and extracts of *Cymbogon citratus* by three different methods, including DPPH method, FRAP assay, and ß-carotene bleaching assay.[83]

F.Pourmorad et al. (2006), carried out a systemic record of the relative antioxidant activity (DPPH) in selected Iranian medicinal plant species extracts, and also determined the total phenolic and flavonoid content by spectrometric methods.[84]

3.9.3. MTT ASSAY

Ali Abdullah et al. (2012), studied the cytotoxicity effect of methanolic extract of *Fenugreek* whole plant and investigated the mechanism involved in its growth-inhibitory effect on MCF-7 human breast cancer cells. The percentage cell viability was calculated based on absorbance read in ELISA reader at 570 nm.[85]

Vidhya and Devaraj et al. (2011), reported the anti –proliferative action of eugenol treated MCF-7 cell lines by MTT assay. The percentage of cell viability was calculated based on absorbance read in ELISA reader at 570nm. [86]

Vidhyalakshmi et al. (2012), investigated the anti-cancer activities of two polysaccharides produced by Bacillus species and Pseudomonas species against MCF-7 cell line and viable cells were determined by the absorbance at 570nm with reference to 655nm.[87]

3.9.4. DNA FRAGMENTATION

Vidhyalakshmi et al. (2012), investigated the apoptosis of human breast cancer cells (MCF-7) induced by polysaccharides produced by bacteria and the result of DNA fragmentation was confirmed by DNA ladder assay.[88]

Vidhya and Devaraj et al. (2011), studied the induction of apoptosis by eugenol in human breast cancer cells. Used various staining technique for observe apoptosis such as giemsa stain for morphological alteration, fluorescence microscopy analysis of cells using acridine orange and ethidium bromide and quantification of DNA fragments. The cell shrinkage, membrane blebbing and apoptotic body formation were observed and reported. [89]

Khaghani et al. (2011), studied the induction of apoptosis in *Hibiscus sabdariffa* treated MCF-7 cell line. The pattern of DNA cleavage as sign of apoptosis was analyzed using a DNA ladder kit by agarose gel electroporosis method.[90]

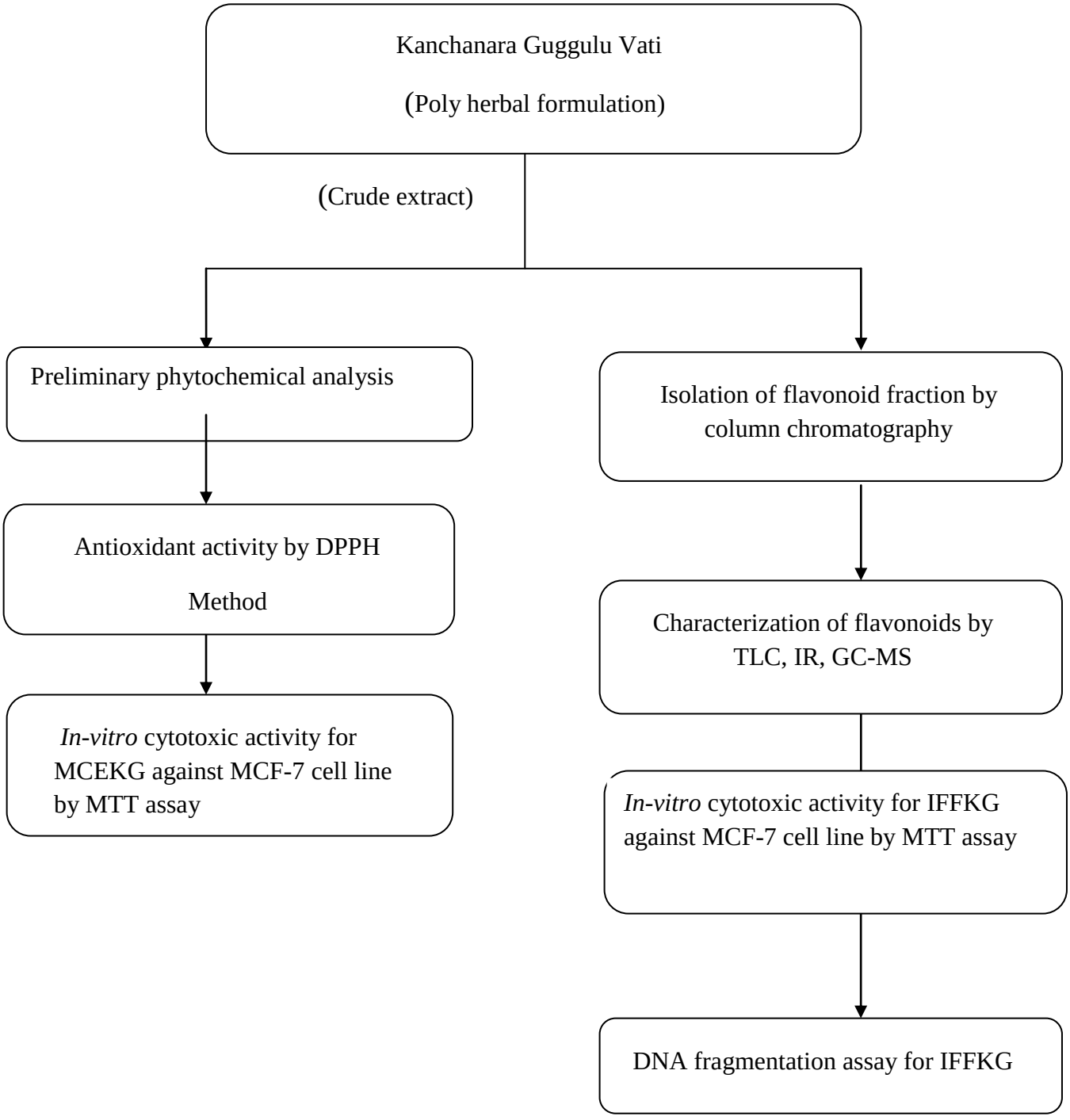
3.9.5. COLUMN CHROMATOGRAPHY AND TLC

Bharathisampantham et al. (2016), isolated the flavonoid fraction from *Trigonella foenum-graecum* leaves extract. The isolated compound were subjected to TLC, FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectroscopy for their characterization. And then free radical scavenging activity was studied for the isolated flavonoid quercetin compound.[91]

Kumuthavalli et al. (2010), isolated the ellagic acid from *Terminalia bellerica* by column chromatography and characterized the isolated compound on the basis of TLC, IR and GC-MS studies. And for the isolated compound anti-cancer activity was studied against DMBA induced papilloma in rats.[92]

Li –jing Lin et al. (2016), isolated and identified the flavonoid components from *Pteris vittata L.* plant extract, and the isolated components were characterized by thin layer chromatography, and NMR studies.[93]

4. SCOPE AND PLAN OF WORK



5. MATERIALS AND METHODS

5.1 PLACE OF STUDY

Phytochemical evaluation, anti-oxidant activity, column chromatography studies are carried out in ARMATS research institute, Maduvangarai, Guindy, Chennai.

MTT assay, DNA fragmentation studies are carried out in LIFETECK research center, Arumbakkam, Chennai.

IR, GC-MASS studies are carried out in IIT University, Chennai.

5.2 INSTRUMENTS USED

- Column chromatography
- UV-spectrophotometer
- Heating condenser
- FT-IR spectrophotometer
- GC-MS analyzer
- Microscope
- Gel electrophoresis
- Co2 incubator

5.3 PREPARATION OF TEST SAMPLE EXTRACT

Kanchanara guggulu vati is an ayurvedic poly herbal formulation, which was purchased from Sarvayush Ayurvedic medicals, Arumbakkam, Chennai.

Direct extraction

Direct extraction with methanol solvent following the method of Eloff, 1998 was used as extraction method, finely grounded kanchanara guggulu (poly herbal formulation tablets)

powder was extracted with methanol in the ratio of 1:10 in conical flask in shaking condition for overnight. The extract was filtered through the Whatmann No: 1 filter paper in a separate container. The process was repeated three times with the same test sample but using fresh solvent. The solvent was removed by placing the extracts in condensation process at 45ºc temperature. The extracted residue were weighed and re-dissolved in methanol solvent to yield 10mg/ml solution and the solution was ready for further analysis.

5.4 PHYTOCHEMICAL ANALYSIS[80, 81]

The following tests were carried out for analyze the phyto-chemicals such as alkaloids, carbohydrates, phenolic compounds, flavonoids, saponins, fixed oils and steroids present in the methanolic crude extract of Kanchanara guggulu vati.

Test for alkaloids:

A small portion of the extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents such as,

1. Mayer's reagent - cream precipitate
2. Dragendroff's reagent - brown precipate

Test for carbohydrates:

The small amount of extract was dissolved in 5ml of distilled water and filtered. The filtrate was subjected to carry out the following tests,

1. **Molish's test:** The filtrate was treated with 2-3 drops of 1% alcoholic α-napthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Brown ring formation between the layers indicates the presence of carbohydrates.

2. **Fehling's test:**The filtrate was treated with 1ml of fehling's solution and heated. Orange precipitate indicates the presence of carbohydrates.

Test for glycoside:

The small amount of extract was hydrolyzed with hydrochlroric acid for few hours in a water bath and the hydrolates was subjected to Legal's test.

Legal's test: Hydrolates was added to with chloroform and the chloroform was then separated. To this equal quantity of dilute ammonia was added. Purple colour in ammonical layer indicates the presence of glycoside.

Test for phenol and tannins:

A small quantity of extract was dissolved in water then ferric chloride solution was added the presence of blue or green colour indicates the presence of phenolic acids.

Test for flavonoids:

A small amount of extract was dissolved in 1ml of distilled water. 0.5 ml of dilute ammonia solution was added to it. Concentrated sulphuric acid was added later. A yellow colour indicated the presence of flavonoids. The yellow colour disappeared on allowing the solution to stand.

Test for saponins:

A small amount of extract was dissolved in 5ml of distilled water and shaken vigorously till a stable persistent froth was obtained. The froth was mixed with 3 drops of olive oil and shaken vigorously and then observed for emulsion.

Test for terpinoids:

Alkaline reagent test: A small amount of extract was treated with few drops of sodium hydroxide solution and observed for intense yellow colouration which disappeared on the addition of dilute Hcl.

Test for lactones:

The extracts treated with sodium picrate solution shows the appearance of yellow to orange colour which indicates the presence of lactone ring.

Test for proteins:

2ml of Biuret reagent was added to 2ml of extract. It was shaken well and warmed on water bath. Appearance of red or violet colour indicates the presence of proteins.

Test for fixed oils and fatty acid:

Spot test: Prepared spot on the filter paper with the test solution and oil staining on the filter paper indicated that the presence of fixed oil & fats.

Test for steroids:

Libermann Buchard test: The solution of 1ml was treated with few drops of acetic anhydride, boiled and cooled, concentrated sulphuric acid was added from the sides of the test tube and observed for a brown ring at the junction of the two layers and green layer in upper layer.

5.5 EVALUATION OF ANTIOXIDANT ACTIVITY [82,83,84]

- The radical scavenging activity of methanolic crude extract was determined by using DPPH assay according to Chang et al., (2008) with small modification.
- The stock solution of extracts were made prepared in methanol to achieve the concentration of 1mg/ml.
- Dilutions were made to obtain concentrations of 20µg, 40µg, 60µg, 80µg, 100µg, and 120µg/ml.
- Diluted solutions (1 ml each) were mixed with 1 ml methanolic solutions of DPPH in concentration of 1 mg/ml.
- After 30 min incubation in darkness at room temperature, the absorbance was recorded at 517nm using spectrophotometer.
- Reference compound being used was ascorbic acid and experiment was done on triplicate.
- Control cuvette contained all the reagents except the test sample extract.
- Percentage inhibition was calculated using equation 1, whilst IC50 values were estimated from the % inhibition versus concentration plot, using non-linear regression algorithm.
- The data were presented as mean values ± standard error mean (n=3).

$$\% \text{ of DPPH radical scavenging activity (\%RSA)} = \frac{\text{Abs. control} - \text{Abs. sample} \times 100}{\text{Abs. control}}$$

Abs. control is the absorbance of DPPH radical + solvent

Abs. sample is the absorbance of DPPH radical + test sample.

5.6 IN-VITRO STUDIES

MTT ASSAY FOR METHANOLIC CRUDE EXTRACT OF KANCHANARA GUGGULU VATI [85,86,87]

The anticancer activity of Kanchanara guggulu was evaluated by MTT assay against Breast cancer cell line (MCF-7).

It is a universally accepted *in- vitro* method for screening the drugs having cytotoxicity activity. It was described by Mosmann (1983) & Monks (1991). This assay is used to determine the IC_{50} of drugs or extracts (concentration of drugs required to inhibit 50% cell growth).

Materials required

- Bio safety cabinet
- UV-chamber
- CO2 incubator
- Inverted microscope
- 96 well micro titre plate
- Fully grown/ confluency reached cell in culture flask
- Minimum essential medium with 10% FBS
- TPVG solution
- Test drug extract (methanolic extract)
- DMSO solution (0.1% v/v)
- Aluminium foil
- Micropipette
- Reagent bottle
- Trypan blue

- MTT Invitrogen
- Penicillin/streptomycin (250 U/ml)
- Gentamycin (100µg/ml)
- Amphotericin B (1 mg/ml)
- Acridine orange

MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazolium bromide) was obtained from Sigma, USA. All other fine chemicals were obtained from Sigma-Aldrich, St. Louis.

Cell line and cell culture

- The Human Breast Cancer Cell line, MCF-7 was obtained from NCCS (National Centre for Cell Science) Pune.
- MCF-7 cells obtained from NCCS were cultured in Rose Well Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, penicillin/streptomycin (250U/ml), gentamycin (100µg/ml) and amphotericin B (1 mg/ml).
- All cell cultures were maintained at 37ºc, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passages weekly and the culture medium was changed twice a week.

Cell treatment procedure

- The monolayer cells detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspension and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1×10^5 cells/ml.

- One hundred microlitres per well of cell suspension were seeded into 96-wellplates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºc, 5% CO_2, 95% air and 100% relative humidity.
- After 24 h the cells were treated with serial concentration of the samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium.
- Additionally seven serial dilution were made to provide a total of eight sample concentrations. Aliquots of 100µl of these different sample dilutions were added to the appropriate wells already containing 100µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37ºc, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

Principle

The Tetrazolium salt, 3-(4, 5-dimethyl thiazole-2-yl)-2, 5, diphenyl tetrazolium bromide is reduced into formazan product by the mitochondrial dehydrogenase enzyme of live or metabolically active cells. The intensity of blue colored formazan produced is directly proportional to the cell viability.[92]

Procedure

- Cells (1×10^5/well) were plated in 24- well plates and incubated in 37ºc with 5% CO_2 condition. After the cell reaches the conditions, the different concentrations (7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000µg/ml) of the test sample and standard 5-fluorouracil were added and incubated for 24 hrs.

- After incubation, the sample was removed from the well and washed with phosphate- buffered saline (p^H 7.4) or MEM without serum.
- 100µl /well (5mg/ml) of 0.5% MTT was added and incubated for 4 hours. After incubation, 1 ml of DMSO was added in all the wells and the absorbance at 570nm was measured with UV- spectrophotometer using DMSO as blank.
- Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

  Percentage cell viability = A570 of treated cells / A570 of Control Cells ×100

  Graphs are plotted using the % cell viability at Y- axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

5.7 COLUMN CHROMATOGRAPHY [89, 90, 91]

Column chromatography is an isolation technique in which the phytoconstituents are being eluted by adsorption.

Russian botanist Mikhail Tswet invented column chromatography in 1906 as a means of studying plant pigments, but it soon became clear that the technique provided a means for separating many complex homogenous mixture into their individual components.

PRINCIPLE

The principle involved in this separation of constituents is **adsorption** at the interface between stationary phase and mobile phase. The component which is having lower affinity towards stationary phase will elute first, and component having higher affinity towards stationary phase eluted last. No two compounds are alike in the above aspect.[93]

5.7.1 ISOLATION OF FLAVONOID FRACTION BY COLUMN CHROMATOGRAPHY

Materials required

- Analytical column (3cm diameter ×75cm ht)
- 15 ml Test tubes (Borosilicate)
- Beaker (50ml)
- China dish
- Glass rod
- Micropipette
- TLC plate
- Cotton
- Test tube stand
- Iodine chamber
- UV chamber

Stationary phase

The most commonly used stationary phase for column chromatography is silica gel, alumina. Here, silica gel G (100-200 mesh size) was used as a stationary phase.

Mobile phase

The mobile phase or eluent is either a pure solvent or a mixture of a solvent used to elute the mixture of components from the complex mixture. Based on the extract nature, solubility, and polarity, the following solvents were used;

- Hexane
- Ethyl acetate
- Methanol

Slurry preparation method

Wet packing method

- The column was filled about one third with hexane
- The measured amount of silica gel was taken in a separate beaker, and it was mixed with hexane (one and half times the volume of silica) by using a glass rod to get the slurry.
- The slurry was slowly poured in to the column by tapping the column gently to encourage bubbles to rise and the silica to settle, then solvent allowed to drain to prevent overflowing.
- The slurry was continuously transfer to the column until all the silica was added. After that, the column was rinsed by draining the solvent at the edge.
- The solvent fully drained until the solvent level was just even with the surface of the stationary phase.

Column running procedure

- The methanol extract was subjected to silica gel column chromatography for the isolation of the flavonoids fraction.
- Since the methanolic extract was found to possess significant pharmacological activity when compared to other extracts an attempt was made to fractionate the methanol extract by column chromatography.
- The elution was done by using solvents of different polarity like hexane, ethyl acetate, and methanol.
- An appropriate column sized 5cm diameter and 50cm length was used. It was washed with water and rinsed with acetone and then dried completely.
- Little pure cotton was placed at the bottom of column up to ¾ level. Methanol extract was mixed with equal amount of graded silica gel until it became free flowing powder.
- When it reached the defined state it was slowly poured in to the column containing hexane solvent with slight movement of stirring by glass rod avoid clogging.
- Little cotton was placed on top of silica gel- extract mixture to get neat column pack. The knob at the bottom was slowly opened to release the solvent.
- The elution was done using hexane, ethyl acetate, and methanol in different ratios like hexane (100%), ethyl acetate (100%), ethyl acetate: methanol (50:50) and methanol (100%).
- As soon as the fractions were eluted, it was analyzed by using readymade TLC plate with suitable solvent system according to the polarity of elute.
- All the fractions were tested for the presence of flavonoids by comparing with the standard quercetin solution by using TLC system and the developed chromatogram was observed under UV at shorter wavelength, and R_f value was calcualated.

5.7.2 CHARACTERIZATION OF ISOLATED FLAVONOID FRACTION

1. THIN LAYER CHROMATOGRAPHY

Principle

Thin layer chromatography is based on **adsorption** principle in which separation depends on the selective adsorption of the components of a mixture on the surface of solid. The stationary phase was in the form of a thin layer adhering to a suitable form of backing material over which the mobile phase was allowed to ascend by capillary action. Traditionally analytical TLC has found application in the detection and monitoring of compound through a separation process.[94]

Materials required

- Readymade TLC plate (stationary phase)
- Beaker 50 ml
- China dish
- Micropipette
- UV chamber
- Iodine chamber

Solvent system used

1. Methanol : ethyl acetate (1 :1)
2. Toluene : ethyl acetate : methanol (1:0.5:0.5)

Procedure

- Solvent system methanol: ethyl acetate (1:1), and toluene: ethyl acetate: methanol (1:0.5:0.5) were prepared and kept aside for saturation.
- Readymade pre coated TLC plates were used, in which the backing material may be either of aluminum foil or a solvent resistant polyester sheet. These sheets are cut into desired size and used for the further analysis.
- Then with the help of micropipette extracts was spotted on TLC plates, which were developed in solvent system tank (TLC chamber).
- The developed chromatogram was observed under UV and it was photographed.
- using the following formula,

$$R_f = \frac{\text{Distant travelled by the solute}}{\text{Distant travelled by the solvent front}}$$

2.SPECTRAL STUDIES

IR spectroscopy

Infra –red spectrum of the fraction obtained from the column chromatography of the ethyl acetate methanolic extract kanchanara guggulu, was subjected for its characteristic functional groups.

Instrument used : Perkin Elmer FT-IR

Method : Neat spectra

Wave number : 4000-450cm^{-1}

GC-MS Spectroscopy

GC-MS spectrum of the fraction obtained from the column chromatography of the ethyl acetate methanolic extract was investigated for its purity and molecular weight to characterized the fraction by its mass and mass of fragments.

5.8MTT ASSAY FOR ISOLATED FLAVONOID FRACTION OF KANCHANARA GUGGULU VATI [85, 86, 87]

The fraction obtained from the column chromatography of the ethyl acetate methanolic extract was identified as its containing flavonoid fraction (Quercetin) by characterization studies such as TLC, IR spectroscopy and GC-MS, so the sample was investigated for its cytotoxity activity against MCF-7 cell line.

Cell line

The Human Breast Cancer cell line, MCF-7 was obtained from the NCCS, Pune.

Procedure

- Cells (1×10^5/well) were plated in 24- well plates and incubated in 37ºc with 5% CO_2 condition. After the cell reaches the conditions, the different concentrations (7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000µg/ml) of the test sample was added and incubated for 24 hrs.
- After incubation, the sample was removed from the well and washed with phosphate- buffered saline (p^H 7.4) or MEM without serum.
- 100µl /well (5mg/ml) of 0.5% MTT was added and incubated for 4 hours. After incubation, 1 ml of DMSO was added in all the wells and the absorbance at 570nm was measured with UV- spectrophotometer using DMSO as blank.

- Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

  Percentage cell viability = A570 of treated cells / A570 of Control Cells ×100

  Graphs are plotted using the % cell viability at Y- axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

5.9 DNA FRAGMENTATION STUDY[86, 87, 88]

This study was carried out to determine the DNA fragmenting ability of both flavonoid fraction and 5- fluorouracil. From the both standard and test sample treated and untreated cells, DNA were extracted using Genei TRIsoln reagent. The supernatant obtained at end of the extraction contained DNA were quantified and observed the fragmentation by 1.2% agarose gel electrophoresis method.

Agarose gel electrophoresis method

Electrophoresis is a technique of separating substances based on the size of the molecule under the influence of an electric field. Bigger molecules moves slowly whereas, smaller molecules move faster.

Agarose gel electrophoresis is a method of separating DNA using flexible gel like slab, under the influence of electric field. It is widely using method for the analysis of nucleic acid and proteins.

Materials required

- Agarose
- TTE buffer
- Bromophenol blue dye
- 10X loading buffer
- DNA ladder
- Extracted DNA
- Electrophoresis chamber
- Gel casting tray and comb
- Conical flask
- Sterile tips and pipette
- Microwave oven
- Centrifuge
- Power supply

Procedure

i) Agarose gel preparation

- The gel casting tray and comb were wiped with ethanol and dried it. To form a mould, two ends of the tray were sealed using tape.
- Weighed 1.2 g of agarose powder and transferred to 500 ml conical flask. Added 100 ml of TTE buffer to the flask, heated the slurry in microwave oven until agarose dissolved and the solution became clear.
- Using insulated gloves the flask was transferred in to a water bath at 55ºc to cool the molten gel.

- ❖ The gel casting tray kept on the table horizontally, the comb was placed at one end of the tray.
- ❖ Then cooled gel solution poured into a mould slowly to avoid bubble formation and allowed it to cool for 30 minutes.
- ❖ Removed the tapes from both the ends and placed the tray in the electrophoresis chamber, which was connected with positive and negative electrode, the comb was taken out carefully and added TTE buffer until it covered the gel surface.

ii) Sample loading

- ❖ Based on quantification done by Nano Drop apparatus, placed equal quantity of DNA, obtained from control, test sample extract and standard treated cells, on a piece of parafilm and added enough TTE buffer to made up the total volume of 10µl each.
- ❖ 2µl of 10X loading dye placed near to the drop of DNA sample and mixed both thoroughly.
- ❖ Slowly load 10µg/ml 100bp DNA ladder and 12µl of sample mixtures into the wells of submerge gel successively.

iii) Running the gel

- ❖ Covered the gel tank with lid, turned on power supply and set about 80 volts.
- ❖ Allowed the DNA to turn until the bromophenol blue dye migrated near to end of the gel.
- ❖ After that turned off the power supply, removed the lid, the gel tray had taken out carefully
- ❖ The gel was placed inside the UV transilluminator to visualize the DNA.

6. RESULTS

6.1 PHYTOCHEMICAL SCREENING:

The Phyto-chemical analysis of methanolic crude extracts of kanchanara guggulu vati (Polyherbal formulation) are presented in **Table 2.**

Table 2: Phyto-chemical analysis

S.no	Phytoconstituents	Inference
1	Alkaloids	Present
2	Tannins and Phenolic compounds	Present
3	Terpinoids	Present
4	Saponins	Present
5	Flavonoids	Present
6	Glycosides	Absent
7	Carbohydrates	Present
8	Lactones	Absent
9	Proteins	Present
10	Fixed oils and fatty acid	Present
11	Steroids	Present

6.2 ANTIOXIDANT ACTIVITY

Antioxidant activity (DPPH method) of ascorbic acid and methanolic crude extract of Kanchanara guggulu as shown in **Table 3. and figure 3.**

Table 3: IC50 value and Percentage inhibition of ascorbic acid and MCEKG

S .no	Concentration (µg/ml)	Percentage inhibition of (std) Ascorbic acid	IC50 value (µg/ml)	Percentage inhibition Of MCEKG	IC50 (µg/ml)
1	20	32.663 ± 0.308	35.37	12.213 ± 0.119	70.98
2	40	51.653 ± 0.253		28.227 ± 0.225	
3	60	65.530 ±0.285		49.093 ± 0.147	
4	80	78.573 ± 0.286		52.503 ± 0.208	
5	100	87.303 ± 0.339		61.380 ± 0.208	
6	120	90.247 ± 0.326		68.090 ± 0.191	

All values are expressed as mean ± SEM for triplicates values

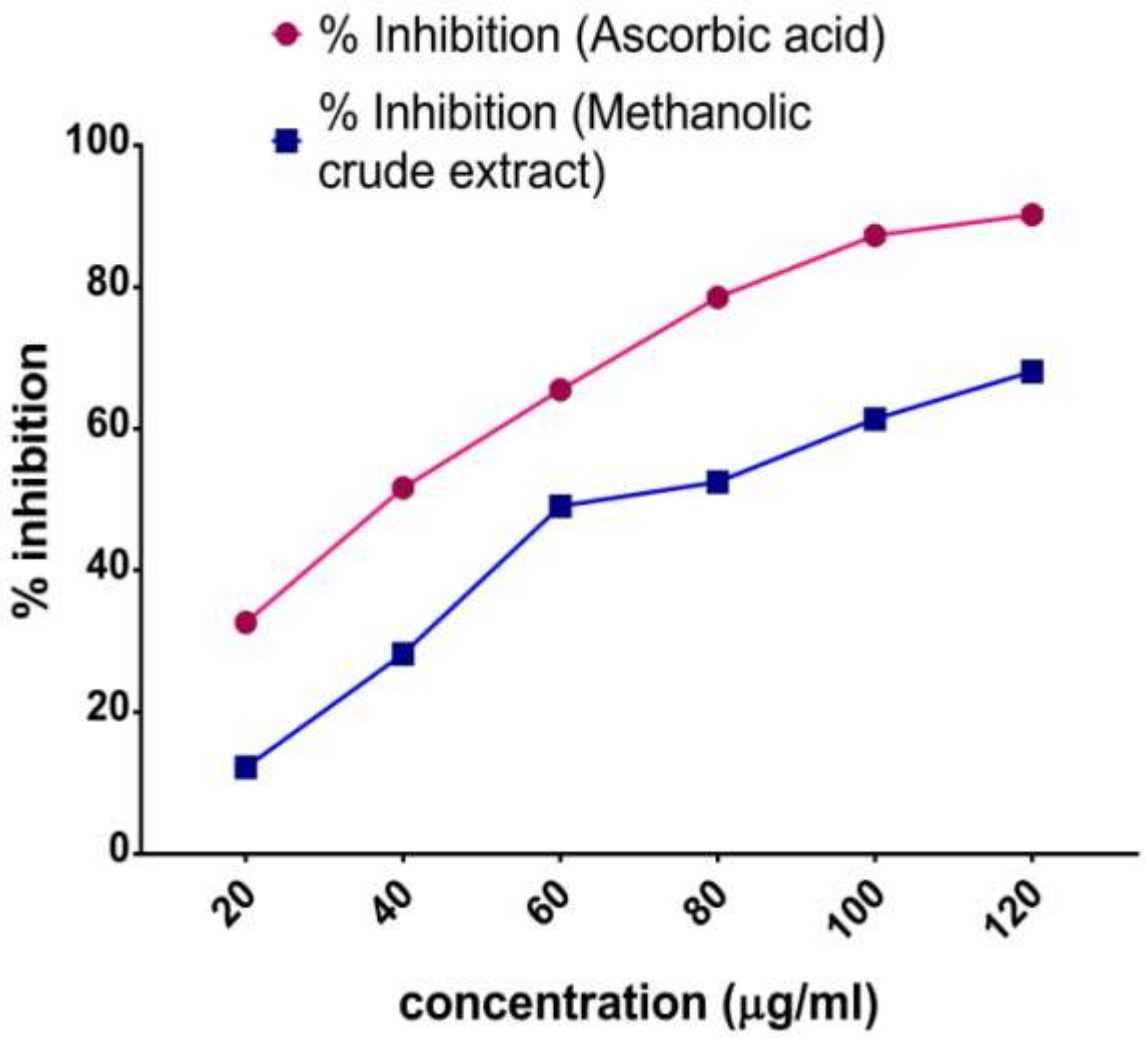


Figure: 3 Percentage inhibition of ascorbic acid Vs MCEKG

6.3 MTT ASSAY

The MTT assay were conducted using 5-Fluorouracil and the methanolic crude extracts of Kanchanara guggulu and the results are presented in **Table 4 & 5 and Figures 4-7.**

Table 4: IC50 value and percentage cell viability of 5- FU

S No.	Concentration (µg/ml)	Absorbance	Cell Viability (%)	IC 50 (µg/ml)
1	1000	0.096	10.24±0.476	15.96
2	500	0.167	17.52±0.265	
3	250	0.225	23.58±0.175	
4	125	0.300	31.70±0.312	
5	62.5	0.370	38.69±0.353	
6	31.2	0.388	40.57±0.337	
7	15.6	0.473	49.46±0.380	
8	7.8	0.545	57.22±0.289	
9	Cell Control	0.954	99.99±0.090	

All values are expressed as mean ± SEM for triplicates values

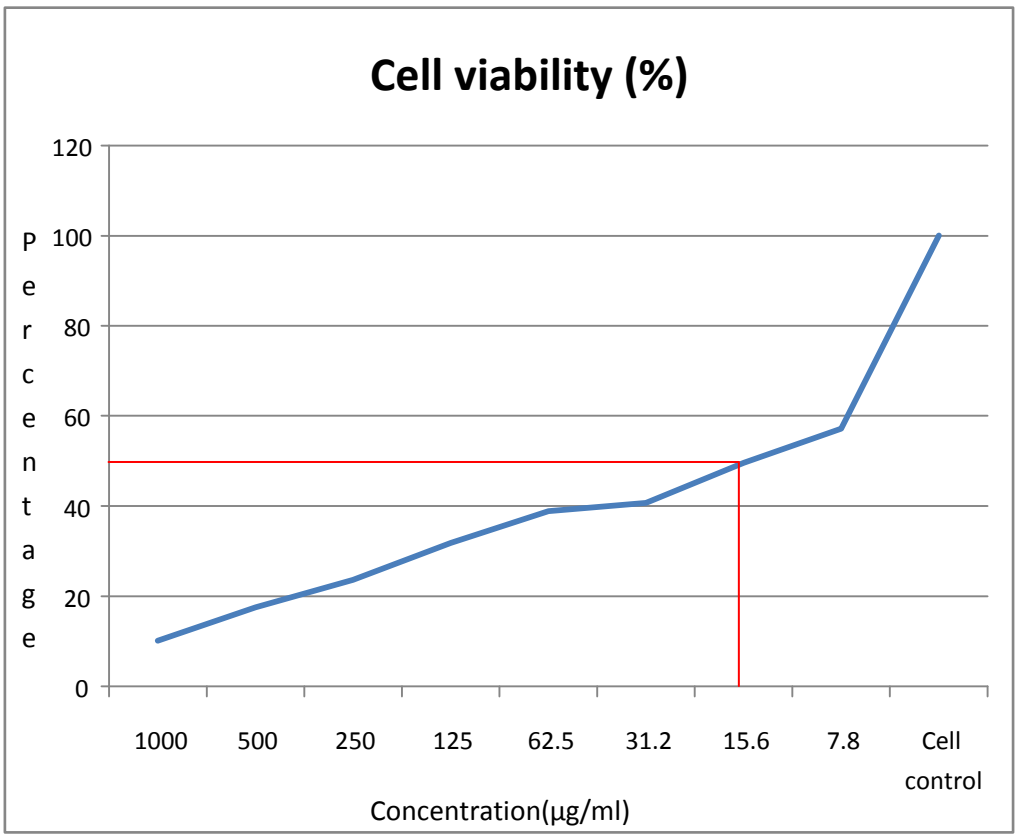


Figure 4. Graphical representation of concentration dependent cell

Inhibition of 5-flurouracil

Anti Cancer Effect of 5-FU on MCF-7 Cell line

Fig. 5a Normal MCF 7 Cell line

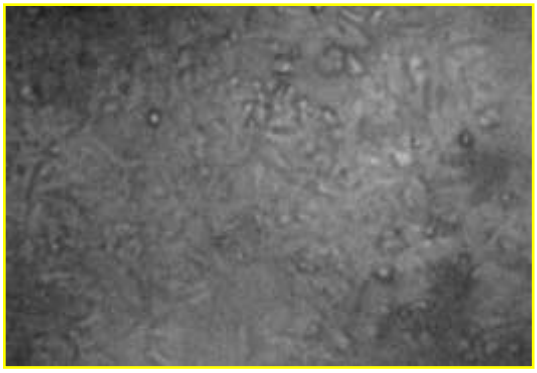


Fig. 5bCytotoxicity - 1000µg/ml

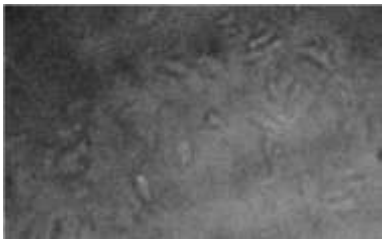


Fig.5cCytotoxicity – 15.6µg/ml

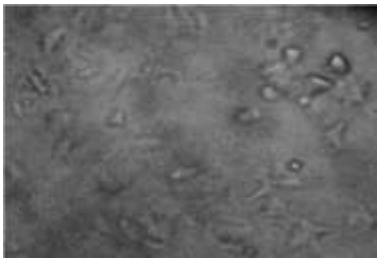


Fig. 5dCytotoxicity – 7.8 µg/ml

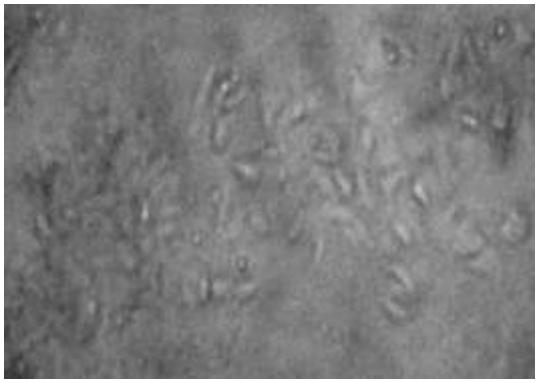


Table 5: IC50 value and Percentage cell viability of MCEKG

S.no	Concentration (µg/ml)	Absorbance	Cell viability (%)	IC50 value (µg/ml)
1	1000	0.09	15.233 ± 0.044	55.26
2	500	0.13	21.976 ± 0.039	
3	250	0.17	28.720 ± 0.165	
4	125	0.21	35.546 ± 0.103	
5	62.5	0.29	49.160 ± 0.118	
6	31.2	0.35	59.346 ± 0.208	
7	15.6	0.41	69.473 ± 0.262	
8	7.8	0.44	74.533 ± 0.292	
9	Cell control	0.59	100.033 ± 0.145	

All values are expressed as mean ± SEM for triplicates values

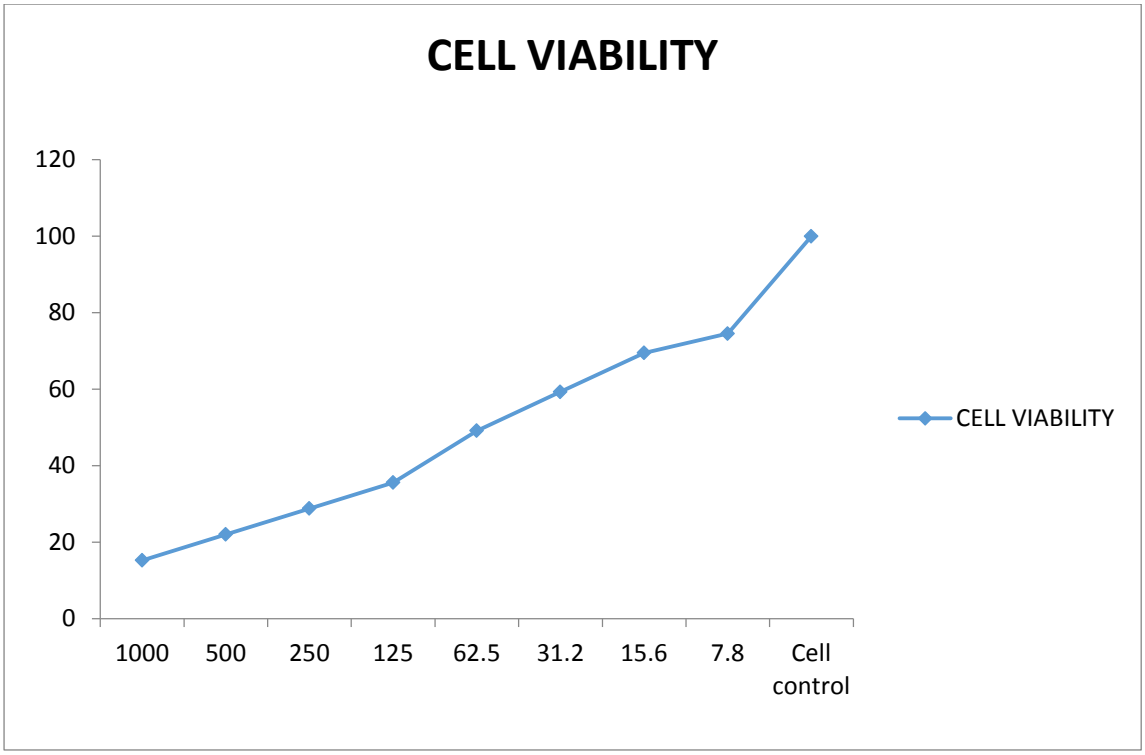


Figure : 6 Graphical representation of concentration dependent Cell viability of MCEKG

Anticancer effect of methanolic crude extract of Kancanara guggulu on MCF-7 cell line

Fig.no:7aNormal MCF-7 Cell line

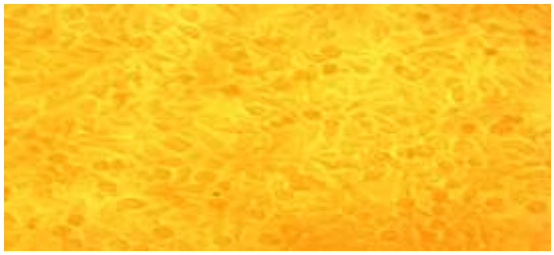


Fig. no: 7bCytotoxicity -1000µg/ml

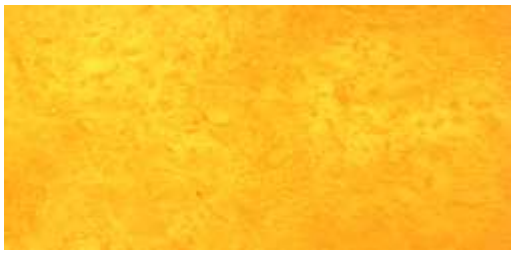
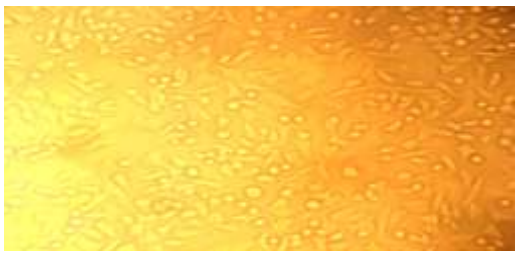


Fig.no. 7cCytotoxicity -250µg/ml

Fig. no. 7dCytotoxicity -125µg/ml

Fig. no. 7eCytotoxicity -62.5µg/ml



6.4 COLUMN CHROMATOGHRAPHY

6.4.1. ISOLATION OF FLAVONOID FRACTION**:**

The methanolic crude extract of Kanchanara Guggulu vati was subjected to column chromatography for the isolation of flavonoid fraction and the results are shown in **Table 6.**

Table 6: Fractions obtained by column chromatography

No. of Fraction	**Eluent**	**Ratio**	**Colour of The fraction**
F1-F2	Hexane	100	No
F3-F4	Ethyl acetate	100	yellow
F3-F9	Ethyl acetate: Methanol	80:20	Light yellow
F10-F11	Methanol: Ethyl acetate	50:50	Dark brown
F12-F13	Methanol: Ethyl acetate	50:50	Light brown
F14-F17	Methanol: Ethyl acetate	50:50	No

6.4.2. CHARACTERIZATION OF ISOLATED FLAVONOID FRACTION:

Thin layer chromatography:

The different fractions obtained from column chromatography are showing different retention factor values. Among the various fractionsno: 10 & 11th determined as best fraction by UV flourescense with three spots. The $R_f$values for each fraction are shown in **Table 7.**

Table 7: R_f values for isolated fraction

Fraction	**Solvent system**	**No. of spots**	**R_f value**
No. 10	Toluene: Ethyl acetate: Methanol (1: 0.5: 0.5)	3	0.717, 0.782, 0.847
No. 11		3	0.777, 0.755, 0.844

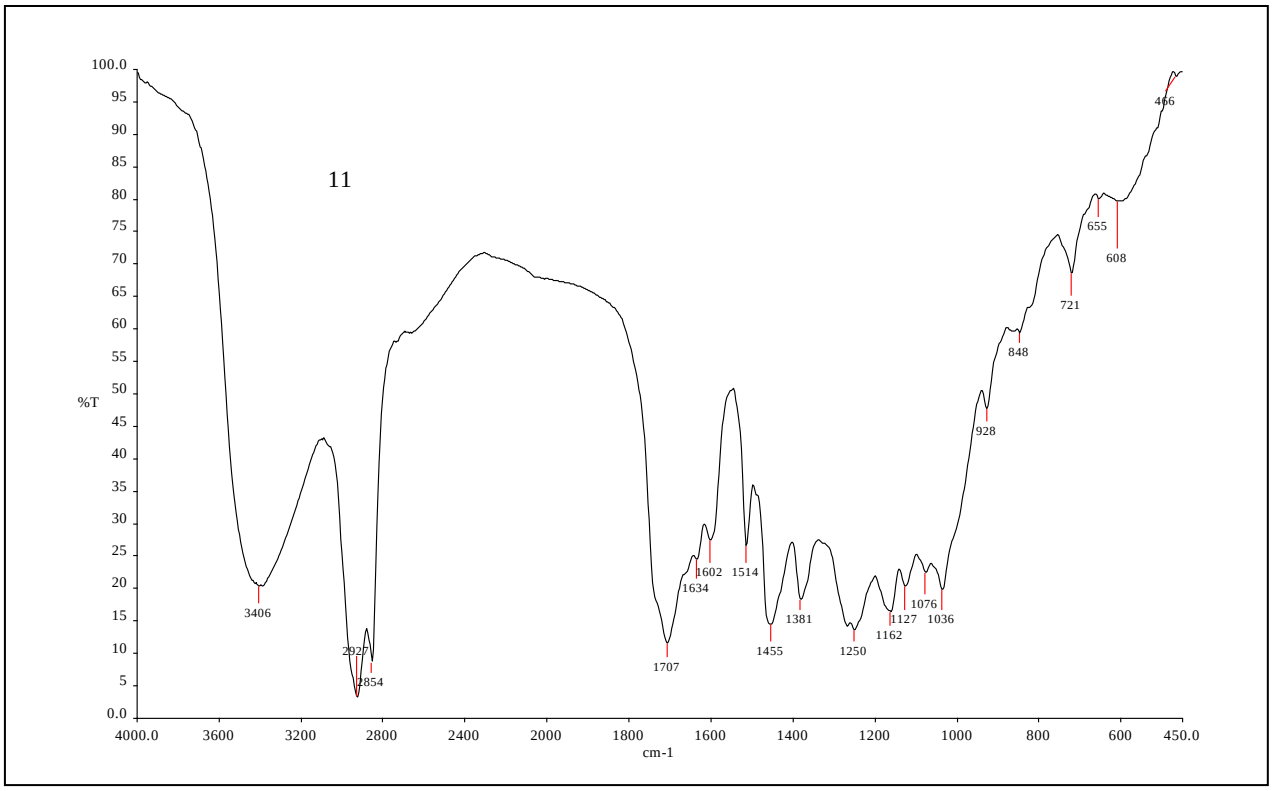


Figure : 8 IR spectrum of isolated fraction

Table 8: IR data for isolated fraction

Wave number	Comments
3406-3200 cm^{-1}	Phenolic OH
2927-2854 cm^{-1}	Ar C-H str
1602-1514 cm^{-1}	Ar C=C str
1707-1634 cm^{-1}	Keto group str (C=O)
863-721 cm^{-1}	Ar C-H bending

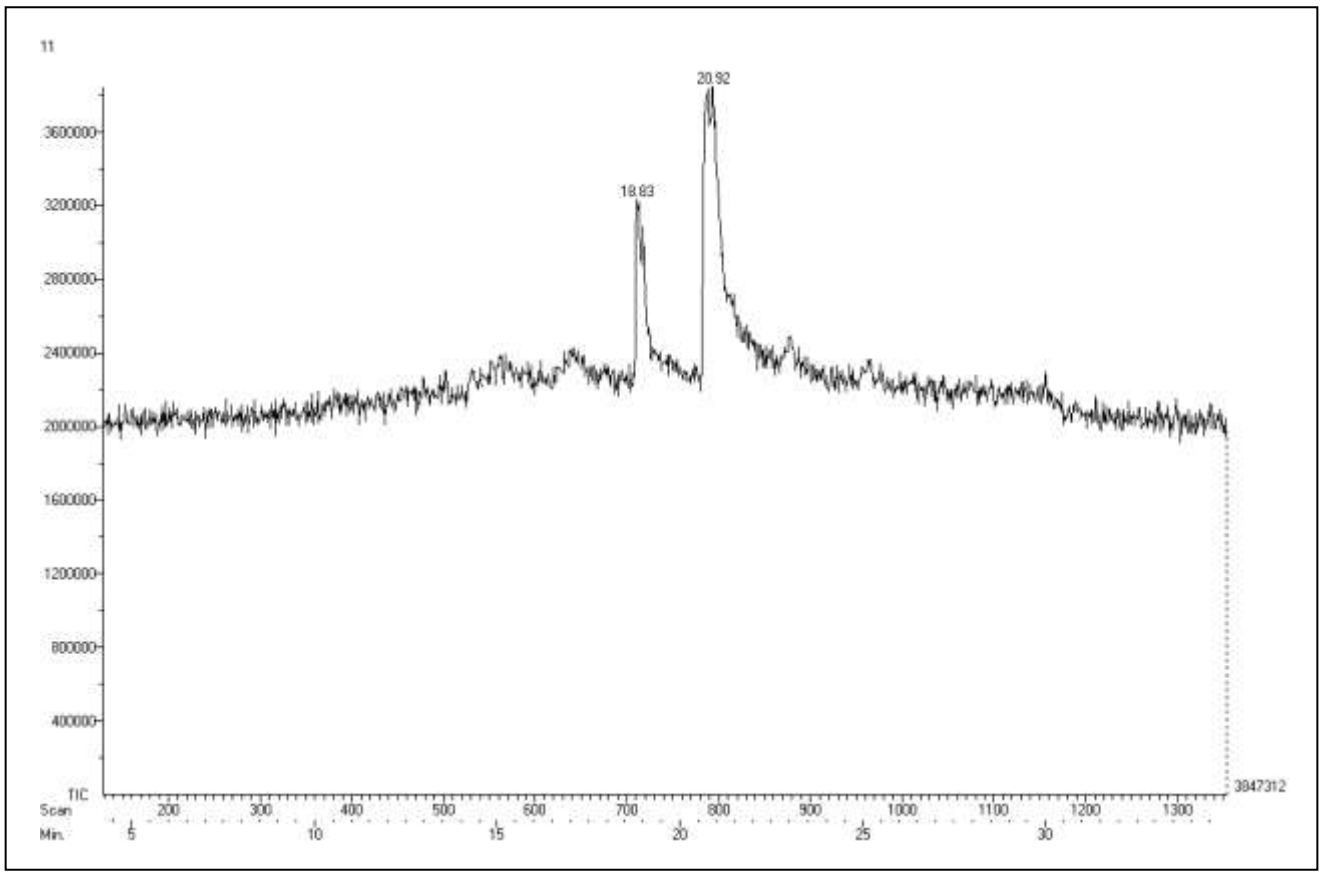


Figure : 9 Gas Chromatography for isolated fraction

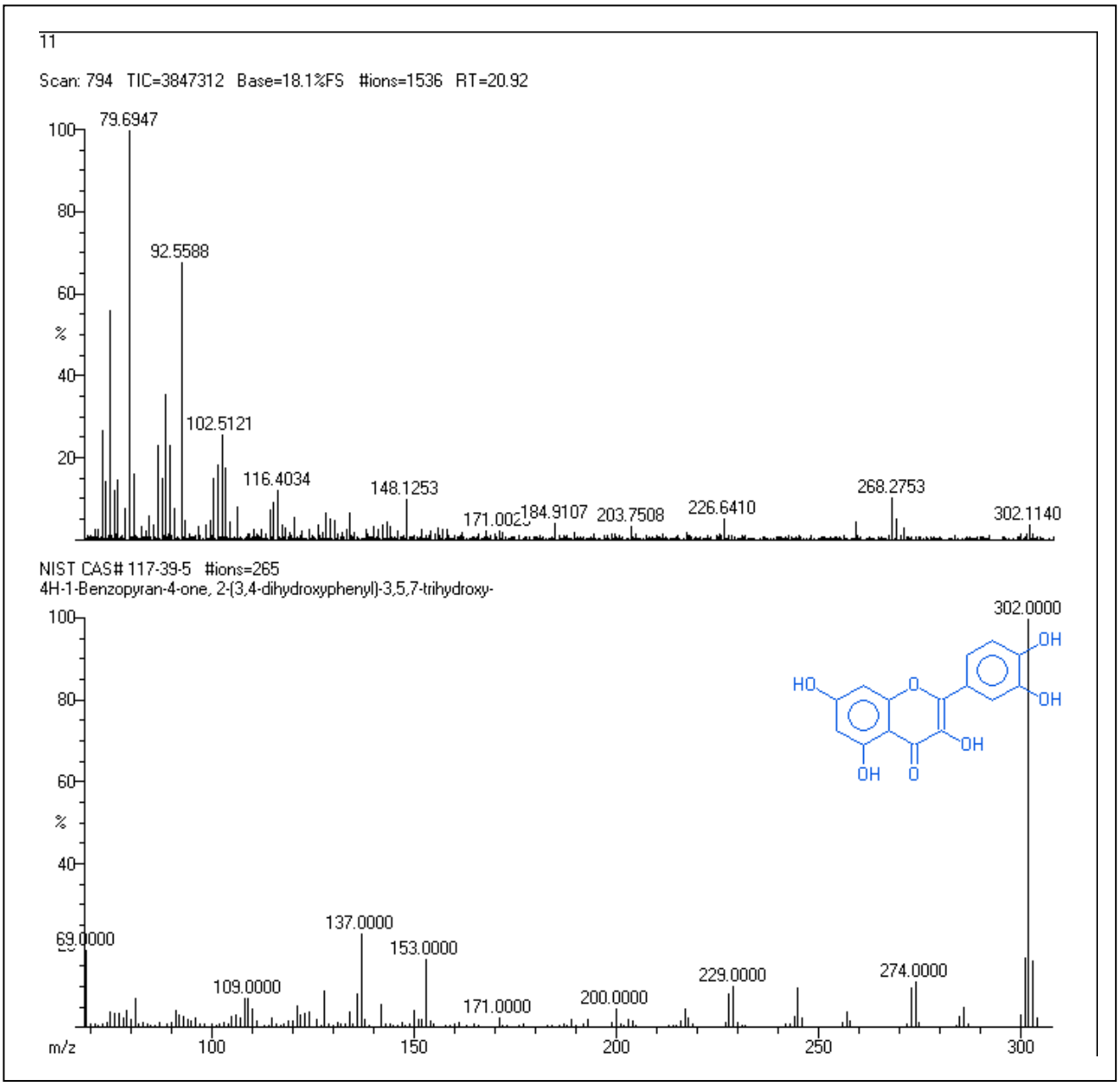


Figure : 10 Mass spectrum for isolated fraction

6.5 MTT ASSAY FOR IFFKG

MTT assay were conducted using isolated flavonoid fraction (no. 11) by column chromatography and the results are shown in **Table 9.& Figure 11-14.**

Table 9: IC50 value and Percentage viability of IFFKG

S.no	Concentration (µg/ml)	Absorbance	Cell viability (%)	IC50 value
1	1000	0.127	13.433 ± 0.292	38.68
2	500	0.203	21.320 ± 0.195	
3	250	0.283	29.600 ± 0.187	
4	125	0.352	36.693 ± 0.269	
5	62.5	0.426	36.203 ± 0.289	
6	31.2	0.497	52.196 ± 0.265	
7	15.6	0.583	60.886 ± 0.596	
8	7.8	0.672	70.566 ± 0.228	
9	Cell control	0.954	99. 793 ± 0.400	

All values are expressed as mean ±SEM values for triplicate values

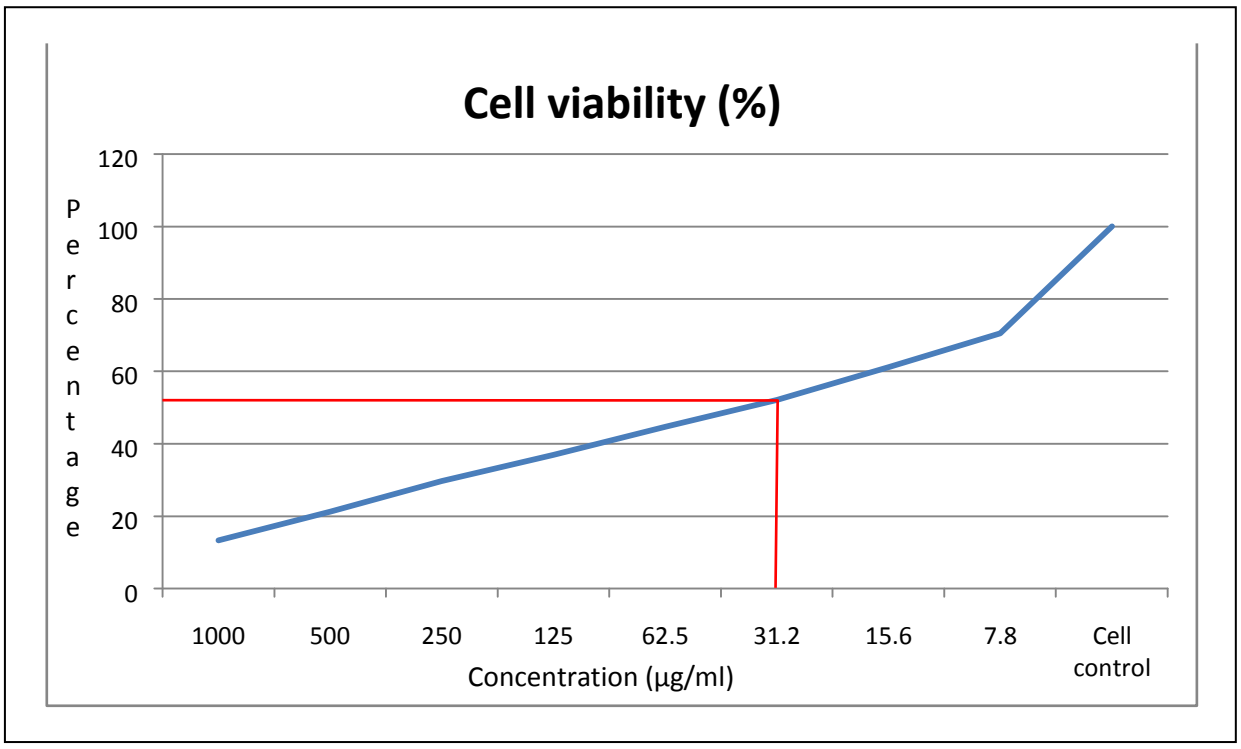


Figure: 11 Graphical representation of concentration dependent cell viability of IFFKG

Anticancer effect of IFFKG on MCF-7 cell line

Fig. no: 12a Normal MCF7 Cell line

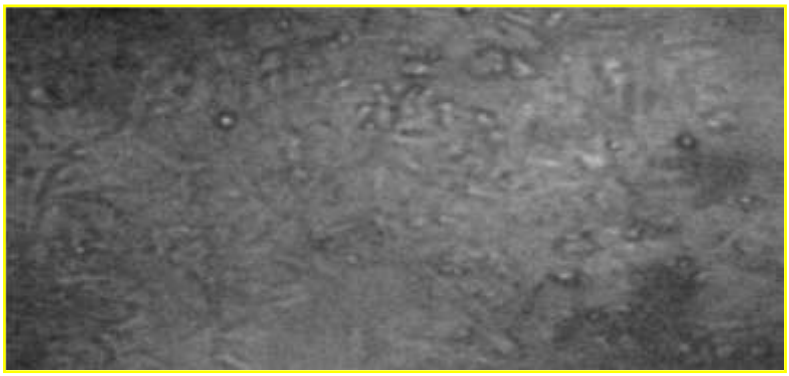
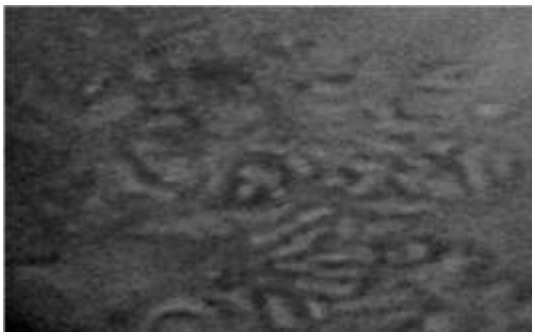


Fig. no12b Cytotoxicity 1000µg/ml

Fig. no 12c Cytotoxicity 31.2µg/ml

Fig.no 12d Cytotoxicity 7.8µg/ml



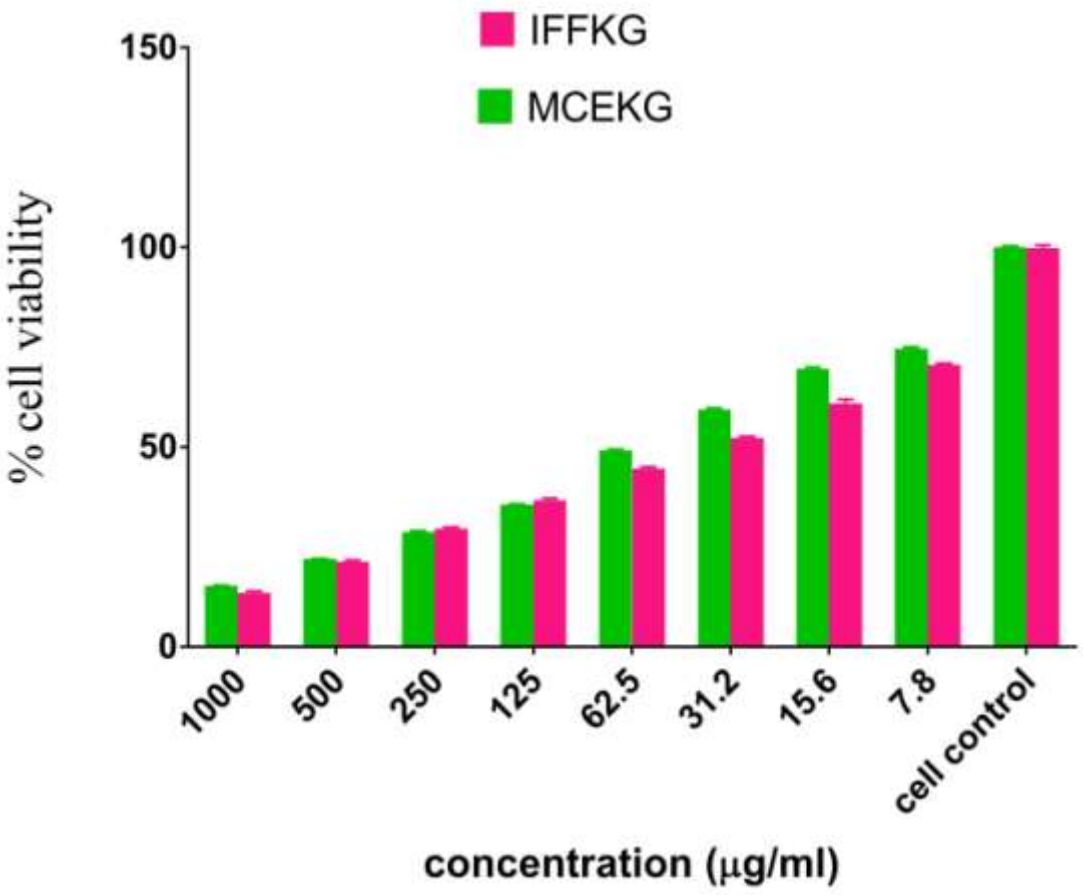


Figure : 13 Percentage cell viability of IFFKG Vs MCEKG

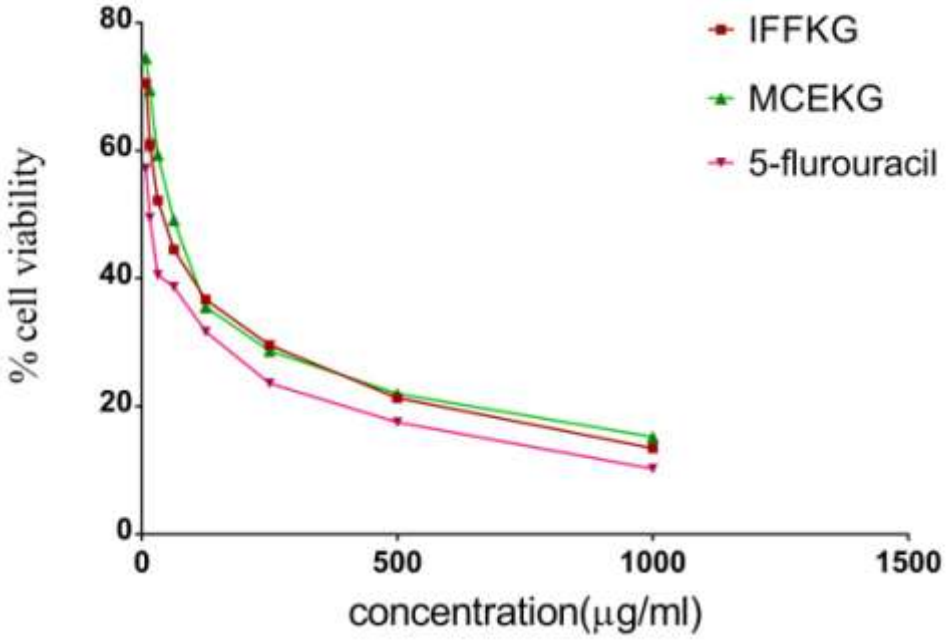
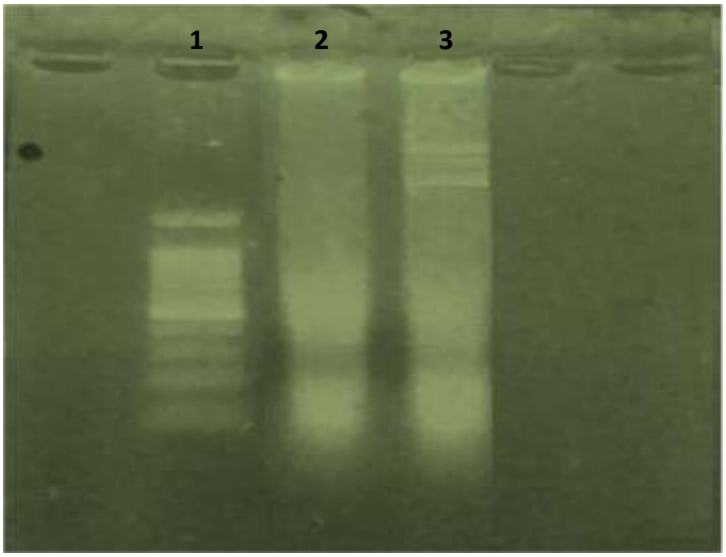


Figure : 14 Percentage cell viability of 5 – FU Vs IFFKG Vs MCEKG

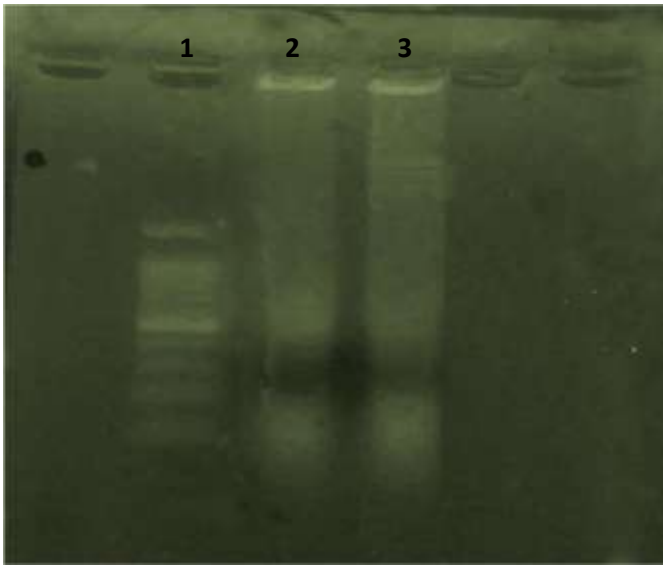
6.6. DNA FRAGMENTATION

The extraction of DNA from the control MCF-7 cells as well as from the treated cells with 5-FU and flavonoid extract were carried out and the results are shown in **Figure 15 & 16.**



Marker control 5-FU

Figure : 15 Fragmentation of DNA



Marker Control IFFKG

Figure : 16 Fragmentation of DNA

7. DISCUSSION

Breast cancer is malignant neoplasm of breast tissue. It is most commonly occurring cancer in females. It accounts for 23% of newly occurring cancer worldwide and represents 13.7% of all cancer death. In both developed and developing countries, breast cancer mortality and incidence rates are projected to rising continuously.[10] With increase in mortality rates among patients suffering from cancer with limited success being achieved in clinical therapies including radiation, chemotherapy, immune modulation and surgery in treating cancer patients, there arises a need for new way for cancer treatment.[95]

Nature has given us a variety of useful medication to cure number of diseases. The role of natural products as a source of remedies for diseases can be dated back to 1500 BC. It has been estimated that 60% of the approved drugs used for treating cancer are derived from natural sources. After long folk practices, many Indian medicines have been screened and they are used for treating and preventing various chronic disorders like cardiovascular diseases and cancer.

Ayurvedic medicines were found to be able to cure diseases better, which were previously not amenable to Western practices. Traditional Indian medicine with its evolution through centuries has always fascinated practitioners and researchers for its applications in a cancer treatment on a scientifically proven research background. Herbal drugs consisting of multiple herb each possessing tremendous potential for a cancer cure are commonly used in Ayurveda. [1]

Kanchanara guggulu vati is an ayurvedic polyherbal formulation which consisting of twelve herbs and it is extensively used for the treatment of hypothyroidism and obesity, PCOS,

tumor, skin diseases and also used as immune system booster, appetite stimulant, and detoxifier.[19]

The phytochemical analysis of methanolic crude extract from Kanchanara guggulu vati revealed that the presence of flavonoids, phenols, tannins, and alkaloids.

7.1 Antioxidant activity

The antioxidant activity of methanolic crude extracts from Kanchanara guggulu vati was evaluated. The largest capacity to neutralize DPPH radicals was found for methanolic crude extract, which neutralizes 50% of free radicals at the concentration of 70.98µg/ml **(Table.3).** In comparison to the IC 50 value of ascorbic acid (35.57µg/ml), methanolic crude extract from Kanchanara guggulu manifested the strongest capacity for neutralization of DPPH radicals. The phytochemical analysis showed that the presence of flavonoid, phenolic compound and tannins .Phenols, and flavonoids are very important plant constituents because of their scavenging activity on free radicals. Therefore, the presence flavonoids, and phenols may contribute directly to their antioxidant activity.

7.2 MTT assay for methanolic crude extract of Kanchanar guggulu vati

In-vitro cytotoxicity activity was carried out in human breast cancer cell line (MCF-7) with the methanolic crude extract and 5-Flurouracil standard drug. The IC_{50} values for 5-Flurouracil and MCEKG was found to be 15.96µg/ml **(Table.4),** 55.26µg/ml **(Table.5)** respectively. Cells treated with higher concentration showed significant decrease in number of viable cells.

7.3 Isolation of flavonoid fraction by column chromatography

The methanolic crude extract from Kanchanara guggulu was subjected to isolation of flavonoid fraction by column chromatography. The sample was loaded and eluted using various extract like hexane (100%), ethyl acetate (100%), ethyl acetate: methanol (80:20), and ethyl acetate: methanol (50:50).

Totally sixteen fractions were collected and each fractions were subjected to TLC studies. Individual fractions was investigated for the presence of flavonoid by comparing with standard quercetin.

Among the various fractions fraction, no: 10 & 11 were determined as best fraction with three yellowish green spots. The R_f value for 10^{th} fraction was found to be 0.717, 0.782, 0.847 and for 11^{th} fraction was found to be 0.777, 0.755, 0.844 **(Table.7).** The two fractions were chosen for further characterization studies.

7.4 Characterization of isolated fraction

The two fractions (10 &11) were analyzed by FT-IR spectroscopy and GC-MS analyzer. From the data 11^{th} fraction was found to be contains flavonoid (quercetin) with 20.92 % **(figure.9)** purity and the molecular weight was found to be 302.11 **(figure.10),** and this fraction was selected for further studies.

7.5 MTT assay for flavonoid fraction obtained by column chomatography

The flavonoid fraction was subjected to MTT assay against human breast cancer cell line (MCF-7) and the IC_{50} value was found to be 38.68µg/ml **(Table.9)**, cells treated with higher concentration showed significant decrease in number of viable cells.

When compared the cytotoxicity effect with methanolic crude extract the flavonoid rich fractions showed the higher cytotoxicity effect with lower IC_{50} value, percentage inhibition was expressed with the help of graph.

This may probably due to the presence of phytochemical flavonoids, because flavonoids have been inhibits the proliferation in cultured cells, the molecular mechanisms of anti-proliferation may involve the pro-oxidant process that causes tumor promotion, also by inhibit ornithine decarboxylase induced by tumor promoters and thus cause a subsequent decrease in polyamine and inhibition of DNA/protein synthesis.[16]

7.6 DNA fragmentation

DNA fragmentation as one of the hallmark of apoptosis was evaluated by gel electrophoresis method. After 48 hours of exposure of MCF-7 cells to IC_{50} concentration of flavonoid fraction of Kanchanara guggulu vati and 5- FU the fragmentation was observed. The untreated cells did not show any fragmentation, which proves apoptosis induced by flavonoid fraction of Kanchanara guggulu vati by fragmenting the DNA **(Figure15, 16).**

8. CONCLUSION

From present study it may be concluded that,

- The phyto chemical investigation revealed the presence of flavonoids, phenolic acids, alkaloids, fixed oils& fatty acids, and steroids, carbohydrates and saponins.
- MTT assay was carried out for methanolic crude extract of Kanchanara guggulu, which inhibited 50% of cell growth at the concentration of 55.26µg/ml.
- The flavonoid fraction was isolated by column chromatography and it was characterized by FT-IR and GC-MS.
- The isolated flavonoid fraction was subjected to MTT assay, which inhibited 50% of cell growth at the concentration of 38.68µg/ml.
- DNA fragmentation was observed in flavonoid fraction treated cells, which confirmed the ability of Kanchanara guggulu induced apoptotic cell death.

The cytotoxicity activity, apoptotic studies which were carried out in extract of Kanchanara guggulu using *in-vitro* methods can be confirmed with *in-vivo* animal models for the future perspective.

9. REFERENCE

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